IMPERIAL INSTITUTE
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AGRICULTURAL RESEARCH, PUSA.

PROCEEDINGS

OF THE

ROYAL SOCIETY OF LONDON

Series B

CONTAINING PAPERS OF A BIOLOGICAL CHARACTER

VOL. CII.

LONDON:
Printed for THE ROYAL SOCIETY and Sold by
HARRISON AND SONS, LTD., ST. MARTIN'S LANE,
PRINTERS IN ORDINARY TO HIS MAJESTY.

April, 1928.

LONDON:
HARRISON AND SONS, LTD., PRINTERS IN ORDINARY TO HIS MAJESTY,
ST. MARTIN'S LANE.

CONTENTS.

SERIES B. VOL. CII.

No. B 713.—August 1, 1927.

PAGE

The Therapeutic Action of some Bismuthyl Derivatives of Organic Hydroxy-Acids. By C. H. Browning, J. B. Cohen, F.R.S., R. Gulbransen, E. Phillis and W. R. Snodgrass (from St. George's House, Medical School, Leeds, the Pathological Department of the University, and the Western Infirmary, Glasgow) 1

A Study of the Direct and Indirect Action of X-Rays upon the Tissues of the Embryonic Fowl. By T. S. P. Strangeways (since deceased) (Lecturer in Special Pathology, Cambridge University), and H. B. Fell, Ph.D. (Beit Memorial Research Fellow). Communicated by Sir William Hardy, F.R.S. 9

The Dynamics of "Sprint" Running. By K. Furusawa, A. V. Hill, F.R.S., and J. L. Parkinson 29

The Energy used in "Spriut" Running. By K. Furusawa, A. V. Hill, F.R.S., and J. L. Parkinson 43

On the Occurrence of the Œstrous Cycle after X-Ray Sterilisation. Part IV.—Irradiation of the Adult during Pregnancy and Lactation: and General Summary. By A. S. Parkes, Beit Memorial Research Fellow. Communicated by Prof. J. P. Hill, F.R.S. 51

Transmission Experiments with Chinese Kala Azar. By E. Hindle and W. S. Patton (Members Kala Azar Commission of the Royal Society). Communicated by Dr. H. H. Dale, Sec. R.S. 63

No. B 714.—September 1, 1927.

Experiments concerning the Question of Secretion of Phenolsulphonephthalein by the Renal Tubule. By A. N. Richards and J. B. Barnwell. Communicated by Dr. H. H. Dale, Sec. R.S. (Plate 1) 72

The Effect of Gamma Irradiation on Cell Division in Tissue Culture in Vitro. By R. G. Canti, M.D., and F. G. Spear, M.B. Communicated by Sir Frederick Andrews, F.R.S. 92

The Distribution of Lymphatics defined by Fatty Acid Compounds developed in the Autolysis of their Contents. By J. L. Smith, M.D., F.R.S., and T. Rettie, D.Sc. (Plate 2) 102

The Mammalian Lacteal: its Histological Structure in Relation to its Physiological Properties. By H. M. Carleton and H. Florey. Communicated by Sir Charles Sherrington, O.M., F.R.S. (Plates 3–5) 110

No. B 715.—November 1, 1927.

PAGE

Measurement of the Biologically Active Ultra-Violet Rays of Sunlight. By L. Hill, F.R.S. (Plate 6) 119

On Intracellular Oxidation-Reduction Potential. By L. Rapkine and R. Wurmser. Communicated by Sir Frederick Hopkins, F.R.S. 128

Bacterial Proteins.—The Presence of Alcohol-Soluble Proteins in Bacteria. By Sir Kenneth Goadby, K.B.E., M.R.C.S., L.R.C.P., D.P.H. Cantab. Communicated by Sir Frederick Hopkins, F.R.S. 137

Absence of a Linear Relationship between Graded Simple Reflex Flexions and the Relaxations thereof evoked by a Constant Extension-Producing Stimulus. By T. G. Brown, F.R.S. (Cardiff) 143

Absence of a Linear Relationship between the Reflex Flexor Shortenings evoked by a Graded Series of Flexion-Producing Stimuli and the "Inhibitory" Lengthenings of a Constant Extension Reflex evoked by the same Stimuli. By T. G. Brown, F.R.S. (Cardiff) 150

The Relation of the Magnitudes of Remaining Reflex Shortening in Two Antagonistic Muscles during Compound Stimulation. By T. G. Brown, F.R.S. (Cardiff) 159

Experiments on the Physiology and Genetics of the Smut Fungi.—Seedling Infection. By S. Dickinson. Communicated by Sir John Russell, F.R.S. 174

No. B 716.—December 1, 1927.

CROONIAN LECTURE.—Organizers in Animal Development. By Hans Spemann, Professor of Zoology, Freiburg i. B. 177

The Enzymatic Function of Mitochondria in the Germination of Cereals. By E. S. Horning, M.A., M.Sc., and A. H. K. Petrie, M.Sc., Biology School, University of Melbourne, Australia. Communicated by Professor A. J. Ewart, F.R.S. (Plates 7–12) 188

The Development of Morphology of the Gonads of the Mouse. Part II.—The Development of the Wolffian Body and Ducts. By F. W. R. Brambell, Lecturer in Zoology, King's College, London. Communicated by Prof. J. P. Hill, F.R.S. (Plates 13–16) 206

Responses to Stimulation of the Motor Area of the Cerebral Cortex. By S. Cooper, Research Fellow of St. Hilda's College, Oxford, and D. Denny-Brown, Beit Memorial Research Fellow. Communicated by Sir Charles Sherrington, O.M., F.R.S. (Plates 17 and 18) 222

Linkage Groups and Chromosome Number in Lathyrus. By R. C. Punnett, F.R.S. ... 236

No. B 717.—January 2, 1928.

Address of the President, Sir Ernest Rutherford, O.M., at the Anniversary Meeting, November 30, 1927 239

The Action of Guanidine on the Melanophores of the Skin of the Frog (Rana Temporaria). By S. Ochoa. Communicated by Prof. D. Noël Paton, F.R.S. (Plates 19 and 20) 256

PAGE

The Formation of Indigotin from Indol by Soil Bacteria. By P. H. H. Gray, M.A., Rothamsted Experimental Station. Communicated by Sir John Russell, F.R.S. (Plate 21) 263

A Consideration of some Attempts to Analyse Growth Curves. By G. E. Briggs, St. John's College, Cambridge. Communicated by Dr. F. F. Blackman, F.R.S. 280

Triplet Children in Great Britain and Ireland. By R. A. Fisher, Sc.D., Rothamsted Experimental Station, Harpenden. Communicated by Sir Arthur Keith, F.R.S. 286

No. B 718.—February 1, 1928.

The Differential Effect of the Ions of Three-Salt Solutions on the Growth of Potato Plants in Sand Culture. By F. G. Gregory, D.Sc. Communicated by Prof. V. H. Blackman, F.R.S. 311

On the Production of Silk by Species of the Genus Hilara Meig. (Diptera). By H. Eltringham, M.A., D.Sc., F.Z.S., with an Appendix on the Habits of the Species, by A. H. Hamm, A.L.S., F.E.S. Communicated by Prof. E. B. Poulton, F.R.S. (Plate 22) 327

A Further Induction of Melanism in the Lepidopterous Insect, Selenia bilunaria Esp., and its Inheritance. By J. W. H. Harrison, D.Sc. Communicated by E. W. MacBride, F.R.S. 338

Induced Changes in the Pigmentation of the Pupæ of the Butterfly Pieris napi L., and their Inheritance. By J. W. H. Harrison, D.Sc. Communicated by E. W. MacBride, F.R.S. 347

Immunological and Serological Studies of the Viruses of Fowl-Pox and Vaccinia. By G. M. Findlay. Communicated by J. A. Murray, M.D., F.R.S. 354

The Air-Resistance to a Runner. By A. V. Hill, F.R.S. 380

No. B 719.—March 1, 1928.

Changes in the Ovary of the Mouse following Exposure to X-Rays. Part IV.—The Corpus Luteum in the Sterilized Ovary and some Concluding Experiments. By F. W. R. Brambell, Lecturer in Zoology, King's College, London, U. Fielding, Demonstrator in Anatomy, and A. S. Parkes, Beit Memorial Research Fellow. Communicated by Prof. J. P. Hill, F.R.S. (Plate 23) 385

Studies in the Microchemistry of the Cell. I.—The Chromatin Content of Normal and Malignant Cells, as demonstrated by Feulgen's "Nuclealreaktion." By R. J. Ludford, Foulerton Research Student of the Royal Society, and Hon. Lecturer in Cytology, University College, London. Communicated by J. A. Murray, M.D., F.R.S. (Plates 24, 25) 397

Cytological Studies on the Viruses of Fowl-Pox and Vaccinia. By R. J. Ludford, Foulerton Research Student of the Royal Society, and Hon. Lecturer in Cytology, University College, London. Communicated by J. A. Murray, M.D., F.R.S. (Plates 26-30) 406

PAGE

On the Chemical Evidences of the Organic Content of Human Enamel. By E. Sprawson, M.C., L.R.C.P. Lond., M.R.C.S., L.D.S. Eng., and F. W. Bury, M.Sc. Vict., A.I.C. Communicated by Prof. W. Bulloch, F.R.S. 419

A Quantitative Study of the Course of Fungal Invasion of the Apple Fruit, and its Bearing on the Nature of Disease Resistance. Part I.—A Statistical Method of Studying Fungal Invasion. By F. G. Gregory, D.Sc., and A. S. Horne, D.Sc. Communicated by Prof. V. H. Blackman, F.R.S. 427

A Quantitative Study of the Course of Fungal Invasion of the Apple Fruit, and its Bearing on the Nature of Disease Resistance. Part II.—The Application of the Statistical Method to certain Specific Problems. By A. S. Horne, D.Sc., and F. G. Gregory, D.Sc. Communicated by Prof. V. H. Blackman, F.R.S. 444

No. B 720.—April 2, 1928.

Experimental Researches on Vegetable Assimilation and Respiration. XVII.—The Diurnal Rhythm of Assimilation in Leaves of Cherry Laurel at "Limiting" Concentrations of Carbon Dioxide. By E. J. Maskell, Emmanuel College, Cambridge. Communicated by Dr. F. F. Blackman, F.R.S. 467

Experimental Researches on Vegetable Assimilation and Respiration. XVIII.—The Relation between Stomatal Opening and Assimilation.—A Critical Study of Assimilation Rates and Porometer Rates in Leaves of Cherry Laurel. By E. J. Maskell, Emmanuel College, Cambridge. Communicated by Dr. F. F. Blackman, F.R.S. 488

The North Chinese Species of the Genus *Phlebotomus* (Diptera, Psychodidæ). By W. S. Patton and E. Hindle (Members of the Kala Azar Commission of the Royal Society). Communicated by Dr. H. H. Dale, Sec. R.S. 533

OBITUARY NOTICES.

Joseph Jackson Lister (with portrait) i
Willem Einthoven (with portrait) v
William Boog Leishman (with portrait) ix
Ernest Henry Starling (with portrait) xvii

Index xxix

PROCEEDINGS OF

THE ROYAL SOCIETY.

Section B.—Biological Sciences.

The Therapeutic Action of Some Bismuthyl Derivatives of Organic Hydroxy-Acids.

By C. H. Browning, J. B. Cohen, F.R.S., R. Gulbransen, E. Phillis, and W. R. Snodgrass (from St. George's House, Medical School, Leeds, the Pathological Department of the University, and the Western Infirmary, Glasgow).

(Received May 4, 1927.)

The chemotherapeutic action of compounds of bismuth in spirochætal infections was observed by Robert and Sauton. These workers found that sodium bismuthyl tartrate had a marked effect in preventing and curing infection with *Spirochæta gallinarum* (Fowl Spirillosis). Since then the action of compounds of bismuth has been extensively investigated in spirochætal infections (see Levaditi). The present work concerns the bismuth derivatives of organic hydroxy-acids. All the compounds investigated are white amorphous solids, of which only the derivatives of the monobasic sugar acids are at all soluble in water. They are stable to acetic acid, but are instantly decomposed by dilute mineral acids; and, with the exception of the citric acid and ester compounds, are soluble in alkali.

The therapeutic action of these compounds was tested in the case of mice infected with *Sp. laverani* (Breinl and Kinghorn; *Sp. muris* of Wenyon). Infections with the strain used, which was non-pathogenic for mice, had the following characteristics:—As a rule, five or six days after subcutaneous inoculation with a suspension in saline of infected blood, the organisms were found in the peripheral circulation in small numbers. Thereafter they usually became more numerous and persisted for long periods. Their abundance in the blood appeared to vary from time to time and after some months they might become very scanty. The value of this infection as a test for chemotherapeutic properties lies in the fact that it is susceptible to agents such as salvarsan, which are effective in the treatment of infections with *Tr. pallidum*.

In the therapeutic experiments a single subcutaneous injection was given, at from one to four weeks after inoculation, when parasites were present in the blood. The blood was then examined almost daily for a month, and subsequently several times a week for other two months; if the organisms failed to be found throughout this period cure was presumed to have occurred. Recurrences were noted as late as 37 days after treatment. The results are shown in the accompanying table.

All the substances produced definite therapeutic action, as shown by rapid disappearance of parasites from the blood. But in most cases the organisms reappeared in the blood, even after doses which closely approached the maximum amount tolerated. No striking differences in the toxicity of the substances were observed; the compound of thiobenzoic acid, however, appeared to be distinctly more toxic than the others. In this connection it should be noted that the bismuth content and the solubility of these compounds varies considerably. Also, in a proportion of animals which received the maximal doses shown in the Table, progressive loss of weight occurred after a time, indicating chronic toxic action.

It has been generally observed that the bismuth compounds hitherto investigated possess chemotherapeutic action in proportion to their content of bismuth, and that alterations in chemical constitution have little influence on this property(Kolle). In the present series of experiments it would appear, however, that the organic constituent of the molecule played a certain part in determining therapeutic efficacy, since the saccharic, mannonic, lactobionic, tartaric, and citric acid derivatives were the only ones which effected cure. The range of effective dosage, however, was extremely small; and with the most active compounds either a proportion only of animals were cured by nearly maximal doses or relapses occurred when slightly smaller amounts were administered.

The action of bismuthyl saccharic acid has been tested systematically in a series of cases of syphilis in the human subject. This compound was selected because of its curative effect in mice infected with *Sp. laverani*. The free acid was administered intra-muscularly, in a 10 per cent. watery suspension, and constituted the sole treatment in 17 cases, 2 of which were in the secondary stage of the disease and the rest of older standing. On the average a dosage equivalent to about 2·5 grams of metallic bismuth was administered to each patient in 6–10 doses at weekly intervals. Definite antisyphilitic action was

Table I.

Compound.	Percentage Bismuth.		Formula.	Tolerated concentration for a 20-gram mouse: 1 c.c. given subcutaneously	Therapeutic effect on mice infected with *Sp. laverani*.	
	Found.	Calculated.			Curative dose.	Dose which failed to cure.
Bismuthyl saccharic acid	63·7	63·25	$C_6H_{10}O_8 . 2BiO$	1/100	1/100 (2 out of 3 animals)	—
Bismuthyl saccharic acid sodium salt	59·2	59·3	—	—	1/100 (1 out of 2 animals) 1/150 (5 out of 11 animals) 1/200 (1 out of 2 animals)	1/400–1/500 (4 animals)
Potassium salt of bismuthyl mucic acid	56·4	56·5	$C_6H_8O_8K_2 . 2BiO$	1/100	—	1/100.
Bismuthyl gluconic acid	50·0	49·6	$C_6H_{12}O_7 . BiO$	1/150	—	1/200 (2 animals). 1/400 (2 animals).
Bismuthyl gluconic acid sodium salt	47·7	47·1	Na = 5·7; calc. 5·5	—	—	1/200.
Bismuthyl mannonic acid	49·7	49·6	$C_6H_{12}O_7 . BiO$	1/200	1/200 (1 out of 2 animals)	1/400*.
Bismuthyl galactonic acid	49·3	49·6	$C_6H_{12}O_7 . BiO$	1/200	—	1/200.
Bismuthyl lactobionic acid	37·25	35·9	$C_{12}H_{19}O_{12} . BiO$	1/50	1/50	1/100.
Bismuthyl maltobionic acid	37·85	35·9	$C_{12}H_{19}O_{12} . BiO$	1/50	—	1/50–1/100.
Bismuthyl bitartaric acid	38·6	38·5	$C_8H_{10}O_{12} . BiO . 2H_2O$	1/200	—	1/250.
Sodium monobismuthyl tartrate	49·5	49·8	$C_4H_4O_6Na_2 . BiO$	1/100	1/100 (2 animals)	1/200.
Bismuthyl monoethyl tartrate	52·2	52·0	$C_6H_{10}O_6 . BiO$	1/200	—	1/250*.
Bismuthyl diethyl tartrate	63·5	63·8	$C_8H_{14}O_6 . 2BiO$	1/200	—	1/200*.
Bismuthyl glyceric acid	20–30	63·0	$C_3H_6O_4 . BiO$	1/200	—	1/250*.

Table I—continued.

Compound.	Percentage Bismuth.		Formula.	Tolerated concentration for a 20-gram mouse: 1 c.c. given subcutaneously.	Therapeutic effect on mice infected with *Sp. laverani*.	
	Found.	Calculated.			Curative dose.	Dose which failed to cure.
Bismuthyl glyceric ester	57·1	57·7	$C_5H_{10}O_4 . BiO$	1/100	—	1/150*.
Bismuthyl gallic acid sodium salt	50·0	49·8	$C_7H_6O_5 . BiO$	1/300	—	1/400.
Bismuthyl malic acid	57·5	58·1	$C_4H_6O_5 . BiO$	1/200	—	1/200*.
Bismuthyl citric acid	50·3	50·0	$C_6H_9O_7 . BiO$	1/200	1/300 (2 animals)	1/400. 1/600 (2 animals).
Bismuthyl glycollic acid	56·8	55·3	$C_4H_8O_6 . BiO$	1/200	—	1/300*.
Bismuthyl dihydroxymalonic acid	57·7	57·9	$C_3H_4O_7 . BiO$	1/300	—	1/300*.
Bismuthyl dihydroxymalonic ester	65·2	65·3	$C_7H_{12}O_7 . 2BiO$	1/300	—	1/400*.
Bismuthyl dihydroxytartaric acid	51·4	51·6	$C_4H_6O_8 . BiO$	1/200	—	1/200*.
Bismuthyl tartronic acid	60·4	60·6	$C_3H_4O_5 . BiO$	1/200	—	1/200.
Bismuthyl thiobenzoic acid	41–44	55·2	$C_7H_5O_2S . BiO$	1/400	—	1/400.

* These substances were not completely dissolved in the concentrations used.

manifested, in the sense that healing of the lesions occurred in 12 cases, including those in the secondary stage. In only one patient was there a distinct change in the Wassermann reaction of the blood serum as a result of such a course of treatment, a positive reaction being replaced by a weakly positive one. It is to be noted, however, that local irritation at the site of injection, as well as general toxic effects characteristic of a slight degree of poisoning due to bismuth, were observed in a considerable proportion of the cases.

Therefore the general conclusion was reached from this series, as well as from the results of a greater number of patients less systematically treated, that this compound from the therapeutic standpoint presents no advantages over the preparations of bismuth in general use (*e.g.*, finely divided bismuth or iodobismuthate of quinine in oily media).

The sodium salt of bismuthyl saccharic acid when injected intra-muscularly in 10 per cent. watery solution was distinctly less well tolerated than the free acid, probably owing to more rapid absorption. Consequently its use was abandoned after a few preliminary trials, as there was no increase in therapeutic action to compensate for the greater toxic effect. This compound was also injected intravenously, but without satisfactory results so far as the effect on syphilitic lesions was concerned.

Bismuthyl gluconic acid has also been administered intra-muscularly and intra-venously in 10 per cent. watery solution. Its antisyphilitic action is marked, but it has not yet been determined whether in this respect it is superior to the bismuth preparations in general use.

Chemical Section.

The bismuthyl compounds were prepared by the general method of treating a solution of an organic hydroxy-acid, or one of its soluble salts, in dilute acetic acid (1 : 4) with a concentrated solution of crystallised bismuth nitrate in dilute acetic acid. If a precipitate formed, the whole was heated to boiling point to aid filtration, the precipitate being filtered off and well washed, first with hot dilute acetic acid and then with water. If no precipitate formed, the mixed solutions were poured into excess alcohol, and the precipitate filtered off and washed with dilute alcohol. In the preliminary stages an excess of hydroxy-acid was always used, but when some idea of the chemical constitution had been obtained, theoretical quantities were employed.

In the case of the esters which would be hydrolysed by treatment as described above, the following method was used: the ester and the requisite amount of bismuth nitrate were dissolved in the minimum amount of glacial acetic acid and left to stand *in vacuo* over sulphuric acid. The bismuth compound separated out slowly and was filtered off and washed, first with dilute acetic acid and then with water, or, if the product was soluble in dilute acetic acid, with dilute alcohol and water. Since these compounds are so insoluble, they cannot be purified, and consequently it is essential that the washing must be thorough. A partial purification of the sodium salts may be effected by repeated solution in alkali and precipitation with alcohol. All preparations were dried *in vacuo* and then in air at 100°. Drying *in vacuo* does not completely remove water from these compounds, but since the residual water is not constant for any particular substance, it cannot be supposed to be water of constitution.

The most characteristic property of these substances is their solubility in alkali. Although they are all, with the exception of the citric acid and ester compounds, soluble in caustic soda solution, the stability of these solutions varies greatly. Bismuthyl saccharic acid is stable in boiling concentrated alkaline solution, while bismuthyl mucic acid is decomposed by warming with dilute alkali. In all cases of alkaline decomposition a hydrated oxide of bismuth is precipitated. A few of the bismuth compounds, notably the monobasic sugar acid derivatives, are soluble in water. They are all insoluble in all organic solvents, with the exception of acetic acid, and are instantly decomposed by mineral acids.

Bismuthyl derivatives of tartaric acid have been known for some time, but since various investigators have apparently obtained different compounds some confusion exists. We have found that the composition of the product depends solely on the method of preparation, several definite compounds having been isolated. By treating a warm solution of bismuth nitrate in dilute acetic acid with a solution of tartaric acid and drying the precipitate at 110° we obtained the compound $BiO \, . \, C_4H_4O_6NO_3$. Rosenheim and Vogelsang prepared a compound $(Bi(C_4H_4O_6)NO_3 5H_2O)$ by treating a nitric acid solution of bismuth nitrate with excess of concentrated tartaric acid solution. Our compound contained 49·5 per cent. of bismuth, compared with the theoretical 49·8 per cent.

When equimolecular quantities of bismuth nitrate and tartaric acid are heated together in acetic acid solution, the product is a white crystalline solid, which does not contain a nitro group, and can be represented by the formula $C_4H_4O_6 \, . \, BiO$.

Found :—Bi,	55·3 per cent. ;	C, 11·9 per cent. ;	H, 1·30 per cent.
Calc. :—	55·7 per cent.	11·8 per cent.	1·34 per cent.

This substance, on solution in dilute caustic soda and precipitation with alcohol, gives the normal sodium salt (found, 49·5 per cent.; calc., 49·8 per cent.). Baudran obtained the acid COOH . CH(OH) . CH(OBiO) . COOH . H_2O by re-fluxing bismuth hydroxide with tartaric acid solution. If an excess of tartaric acid is used and the above reaction carried out in a boiling solution, the crystalline product agrees on analysis with the compounds obtained by Rosenheim and Vogelsang who prepared it by boiling the 49·8 per cent. acid with tartaric acid, and by Telle, who tried to repeat Baudran's work (found, 38·6 per cent. Bi; calc., 38·5 per cent.).

On treating the mono- and di-bismuthyl acids with hot concentrated caustic soda solution, a new salt is formed, which contains 68·4 per cent. of bismuth. This substance has recently been prepared by Giemsa, by digesting bismuth hydroxide with disodium tartrate solution, and according to him may be represented as COONa . CHOBiO . CHOBiO . COOBiO . $4H_2O$ (68·5 per cent. Bi). The corresponding potassium salt was prepared by Rosenheim and Vogelsang by dissolving the bi-tartaric acid in concentrated potassium hydroxide solution.

The bismuthyl compounds generally may be considered to be definite chemical compounds, rather than loose molecular combinations, because, except in the case of the complex tartrates, alterations in the conditions of reaction and in the amounts of reagents used do not alter the composition of the resulting product, which may be represented by a simple empirical formula. Any general formula for this series of compounds should satisfy the following conditions:—

(1) On cautious hydrolysis bismuth is split from the complex and appears as a hydrated oxide. This is taken to indicate that bismuth is combined as oxide rather than as metal in these compounds.

(2) The bismuth is probably combined in a non-ionisable form, since it does not give the usual reactions for bismuth ions.

(3) The formula should explain the following:—

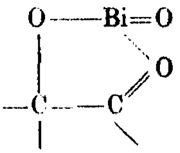
(*a*) The inability of alcohols containing one or more hydroxyls to react.

(*b*) The increased reactivity of hydroxy esters compared with that of the corresponding acids.

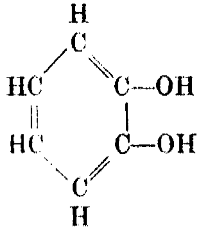
(*c*) The possibility of preparing bismuth derivatives in which one or two hydroxyls take part in the reaction.

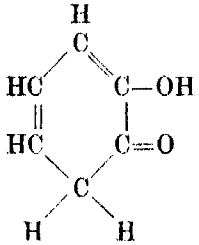
The structure put forward by Maschmann for bismuthyl derivatives of alizarin appears to satisfy the above conditions more nearly than any other.

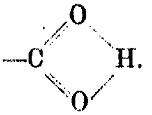
On this view, the structure of the bismuthyl series may be represented by a general formula



This formula satisfies conditions (1) and (2), and (*a*) and (*b*) of condition (3). Alcohols cannot react, because they do not contain the requisite ketone group. In the case of o-dihydroxy phenols this might be formed by a single tautomeric change:—





The carboxyl group is generally supposed to be best represented by a dynamic formula, such as  In this state the ketone group is not freely available, but when the acid is esterified the possibility of tautomerism is removed and the ketone group will be fully available.

No explanation can be given to account for the observation that sometimes one and sometimes two hydroxyl groups appear to take part in the reaction. Certain α-hydroxy-acids will not give bismuthyl derivatives under the experimental conditions investigated. This may be due to steric effect. Glycollic acid readily gives a bismuthyl derivative. Lactic acid (methyl glycollic acid) reacts to give a substance of indefinite composition. No reaction has been obtained with mandelic acid (phenyl glycollic acid), α-hydroxy iso-butyric acid (di-methyl glycollic acid), or with benzilic acid (diphenyl glycollic acid).

Analysis.—Analysis was usually carried out by estimating the amount of bismuth present. 0·2–0·5 gr. of the substance was dissolved in dilute hydrochloric acid and the bismuth precipitated from the hot solution with hydrogen sulphide. The precipitate was allowed to settle, filtered off, washed with hot water and then with alcohol, and finally dried at 110–120°.

We wish to thank Prof. Warrington Yorke, of the University of Liverpool, for the strain of *Sp. laverani* used in this investigation; the Medical Research Council, with whose support the work was carried out; the Royal Society for a grant which has partly defrayed the cost of the chemical section, and the Department of Scientific and Industrial Research for a grant which has enabled one of us (E. P.) to take part in the research.

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A Study of the Direct and Indirect Action of X-rays upon the Tissues of the Embryonic Fowl.

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(Communicated by Sir William Hardy, F.R.S.—Received May 27, 1927.)

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Introduction.

The experiments here recorded had three main objects :—

(*a*) To investigate the effects of X-rays upon chick embryos of different ages irradiated *in ovo* (Part I).

(*b*) To discover how far the destructive effect of radiation upon the tissues is due to a direct action of X-rays upon the individual cells, and how far it may be attributed to an indirect action resulting from physiological disturbance in the organism (Part II).

(*c*) To determine if possible the nature of such indirect action (Part III).

The authors are indebted to the Medical Research Council, by whom the expenses of these investigations were defrayed.

Previous Work.

During the last four years researches have been carried out in these laboratories upon the action of X-radiation upon cells growing *in vitro*. By using tissue cultures as experimental material it was possible to determine the direct effect of X-radiation upon growing cells apart from indirect effects due to the irradiation of the organism as a whole (Strangeways and Oakley, 1923; Strangeways and Hopwood, 1926).

The action of X-rays upon developing chick embryos has been studied by several observors: Gilman and Baetjer (1904), Bordier and Galimard (1905),* Gaskell (1911), Colwell, Gladstone and Wakeley (1922, 1926), Ancel and Vintemberger (1924), Regaud, Lacassagne and Jovin (1925). It was found that irradiated embryos exhibited retardation or inhibition of development, and degenerative changes (Regaud, Lacassagne and Jovin (1925) and Colwell, Gladstone and Wakeley (1926)) were observed in the tissues. Mitosis was also affected and Gaskell (1911) showed that after exposure the number of mitotic figures at first underwent a progressive diminution; if, however, the dose had not been too severe this diminution was only temporary and after an interval cell division regained its normal rate. Regaud, Lacassagne and Jovin (1925) also described a temporary cessation of cell division in irradiated embryos, but found that after larger doses mitosis, when it reappeared, was for the most part abnormal.

Material and Methods.

Material.—The eggs employed for the first series of experiments were from pure bred White Leghorn stock; for the second set of experiments eggs of Rhode Island red fowls were employed as the White Leghorn eggs were no longer available. The eggs were incubated for three different periods before being irradiated: (*a*) 20–25 hours, (*b*) 6 days, and (*c*) 17 days.

Radiological Technique.—Exposure to X-rays was made in a special electrically heated incubator kept at a constant temperature of 38° C. and regulated by a Hearson's capsule. In the top of this special incubator a circular hole was cut which during exposure was filled with a sheet of transparent celluloid. The eggs of 6 day and 17 day incubation were trans-illuminated before irradiation, and in the case of the 6-day embryos the approximate position of the developing chick in the egg was marked on the shell. Four eggs were exposed simultaneously.

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* Not inspected; quoted by Colwell and Russ (1920).

The eggs were arranged in a small cardboard box, with their long axes vertical and their larger ends uppermost, and were placed immediately beneath the circular window in the top of the incubator. The beam of X-rays was so adjusted that the central ray fell normally and symmetrically on the window.

The exposure or incident dose given was estimated by a Friedrich's iontoquantimeter. The unit employed and designated by 1 e, is the amount of energy of a beam which will produce in 1 c.c. of air under normal conditions, an amount of ionisation that as a saturation current will cause a change of 1 electrostatic unit of quantity of electricity in the measuring system.

The X-ray installation used consisted of an induction coil with mercury interrupter and a tungsten target Coolidge tube. The following conditions were kept standard throughout the investigation : Alternate spark gap (point and plane), 15 cm. ; filter, 2 mm. celluloid ; focal distance to base of egg, 25 cm. ; dose was given at the intensity of 5 e per minute.

In the first series of experiments a dose of 150 e (Friedrich's unit) was given to each batch of eggs. In the second set of experiments the eggs received a dose of approximately 270 e.*

It was impossible to ensure that the younger embryos each received the same dose as the position of the chick in the shell is different in the case of each egg. This unavoidable experimental error, however, was not of material importance since the experiments were concerned with the qualitative effects of X-radiation upon chick embryos rather than with the precise effect of a specific dose.

Histological Technique.—The embryos used for histological examination were fixed in Allen's modification of Bouin's solution, which gave good results. The 20–25-hour and 6-day specimens were fixed whole whilst from the 17-day specimens small representative pieces of tissue were removed for fixation. Sections were stained with hæmatoxylin and eosin and iron hæmatoxylin.

Culture Technique.—Cultures were made by the usual coverslip method, the details of which it is not proposed to set forth in this communication as the technique employed has already been fully described elsewhere (Strangeways, 1924). Cultures were fixed in acetic alcohol and stained with iron hæmatoxylin. The number of mitotic figures in each culture was counted.

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* The absorption of the incident X-rays by the egg-shell was measured under the conditions of the experiment and found to be approximately 40 per cent.

Part I.

A.—*Experiments on 20–25-hour embryos.*

Object of Experiments.—To investigate the effects of radiation upon the tissues of embryos in which the *circulation is not yet established.*

Technique.—The eggs were irradiated by the method detailed above and doses of 150 e or 270 e were given. Embryos were removed for histological examination (*a*) immediately after irradiation, and (*b*) after irradiation, and subsequent incubation at 38° C. for periods ranging from 80 minutes to 6 days.

Results.—Essentially the same results were obtained with both doses although the heavier dose caused rather more pronounced changes in the tissues than the lighter dose.

(*a*) Embryos fixed *immediately* after exposure. The macroscopic appearance of the embryos was unchanged. Sections showed a great reduction in the number of mitotic figures as compared with the normal, but the tissues were otherwise unaltered.

(*b*) Embryos fixed after 80 *minutes'* incubation following exposure. As before the macroscopic appearance was unchanged. Histological examination on the other hand showed more marked changes in specimens fixed after 80 minutes' incubation than in those which had been fixed immediately after exposure. Mitosis was very rare, only occasional isolated figures being found; broken down cells had appeared in the tissues and were sometimes very numerous in specimens irradiated with the larger dose.

(*c*) Embryos fixed after 24 *hours'* incubation following exposure. Inspection with the naked eye showed that the embryos were larger than those fixed immediately or 80 minutes after exposure, but were considerably smaller than the unirradiated controls. Sections showed that mitosis had returned and was abundant, whilst morphological development though distinctly subnormal as compared with the controls had advanced markedly. A varying proportion of the mitotic figures in 10 of the 12 embryos fixed were abnormal showing pluripolar spindles, fragmentation of the chromosomes and other pathological changes similar to those described by Strangeways and Oakley in irradiated tissue cultures. Other forms of cell degeneration (increase in chromatin content, nuclear and cellular disintegration, etc.) were also observed in all the specimens, in most cases on a very extensive scale and in places the mesenchyme was œdematous.

(*d*) Embryos fixed after 3–6 *days'* incubation following exposure. (In these experiments only the larger dose of 270 e was used.) With one exception these specimens showed little and sometimes no advance in development over those fixed after 24 hours' incubation. They also showed a pronounced tendency towards cyst formation, and were sometimes represented by one or more transparent cysts usually containing a certain amount of blood. Mitosis was present in the walls of these cysts, and the remains of various organs, especially of the central nervous system, were generally recognisable, although the tissue was usually very degenerate. The cysts appear to have arisen partly by œdema of the mesenchyme and partly by the distension of the blood-vessels and amnion. Embryos which had not become cystic usually showed advanced necrosis. In two cases no embryo could be distinguished although a fairly extensive vascular area was present. In the exceptional case referred to above recovery appeared to have been complete and a normal living embryo was present.

Summary of Results.—From the above experiments it was seen that if 20–25-hour embryos were fixed and stained *immediately* after irradiation, the only effect observable was a great reduction in the number of mitotic figures present. After 80 *minutes'* incubation mitosis was almost absent and numerous degenerate cells were seen. After 24 *hours* the tissues had recovered to some extent; development had advanced and sections showed that there was a variable amount of abnormal mitosis, but cell degeneration was usually extensive. In specimens fixed after 3–6 *days'* incubation following irradiation necrotic and cystic changes were conspicuous, but sections showed that mitosis was frequently present up to the sixth day of incubation.

B.—*Experiments on 6-day embryos.*

Object of Experiments.—To investigate the effects of radiation upon embryos in which *the circulation is established.*

Technique.—Irradiation was carried out as in the case of the 20–25-hour embryos, doses of 150 e or 270 e being given. Embryos were fixed (*a*) immediately after exposure, and (*b*) after exposure followed by an incubation period ranging from 26 minutes to 24 hours.

Results.—(*a*) Embryos fixed *immediately* after exposure. Embryos from eggs which had received 150 e appeared normal to the naked eye when removed from the shell. Sections showed a great reduction in the number of mitotic figures as compared with the control; a very small number of degenerating cells were present but the tissues were otherwise unaltered.

Embryos which had been exposed to the larger dose (270 e) showed more pronounced changes. Macroscopical inspection often showed reddening of the surface and on histological examination, degenerate cells were found to be quite numerous whilst mitosis was rare or absent. This difference between the effects of the two doses is probably due to the fact that the heavier doses took nearly 25 minutes longer to give, so that the degenerative changes had more time to appear before the end of the exposure. This suggestion was supported by the results of the experiment recorded below, which indicated that in the case of the smaller dose there was a latent period of about 26 minutes before marked degeneration was seen on sectioning.

Embryos fixed after 26 *minutes'* incubation following exposure. Of 3 embryos from eggs exposed to 150 e and fixed after 26 minutes' incubation following irradiation 2 appeared normal on macroscopic examination, whilst in the third the blood-vessels appeared distended, especially in the posterior dorsal region and in the limbs. Sections showed a considerable number of degenerate cells in two of the specimens and a few in the third. Great attention was paid to the walls of the blood-vessels and in two of the specimens—one of which was normal in macroscopic appearance whilst the other showed distended vessels as described above—large numbers of breaking down cells were found in the delicate vascular walls. Mitosis was rare or absent.

Embryos fixed after 52 *minutes'* incubation following exposure. The eggs received a dose of 150 e. Two of the three specimens fixed showed large dark crimson blotches on the head and trunk; in the third individual the vessels were only slightly dilated. In the two specimens showing crimson colouration histological examination revealed pronounced cellular degeneration in all the tissues and extensive extravasation of blood. The specimen of normal external appearance resembled the embryos fixed after 26 minutes' incubation in that there was no extravasation of blood, whilst degenerative cells were present in all the tissues including the walls of the blood-vessels. Mitosis was not observed in any of the specimens. The heart was beating in all the embryos.

Embryos fixed after 80 *minutes'* incubation following exposure. After this period of incubation following irradiation the embryos presented a very striking appearance; both doses (150 e and 270 e) gave the same results. When removed from the shell the chicks were seen to have changed to a dark crimson colour which presented a remarkable contrast to the pale pink flesh of the unirradiated controls; the heart was usually still beating although the pulsations were abnormal. Histological examination showed marked changes. Enormous numbers of degenerate cells were seen in all the tissues and were especially conspicuous in the retina, central nervous system and mesenchyme. Mitosis was either rare or absent. The crimson colour of the embryos was found to be due to the engorgement of the superficial vessels, the walls of which had in many places broken down and liberated blood into the surrounding tissues. This engorgement and extravasation was particularly well seen in the case of the choroid vessels of the eye.

The circulatory disturbance might be explained as follows. A certain proportion of the cells of the embryo were directly affected by the action of the rays, and after a latent period of variable length this resulted, as described above, in the disintegration of the affected cells. The breaking down of many of the cells composing the walls of the blood-vessels might be supposed to result in the liberation of fibrin ferments and the blocking of the blood-vessels with blood clot. This would arrest the circulation, but as the heart was still beating the blocked vessels would become at first congested and then in places ruptured, with the result that blood would be extravasated into the tissues.

Embryos fixed after 5 *hours'* incubation following exposure. Embryos incubated for 5 hours after exposure *in ovo* to 150 e were similar macroscopically and histologically to those incubated for 80 minutes, except for the fact that the number of degenerate cells had increased and some of the tissues had begun to disintegrate ; mitosis was absent except in one specimen in which a few abnormal figures were seen.

Embryos fixed after 24 *hours'* incubation following exposure. With a view to determining whether the 6-day old embryos recovered from the effects of irradiation, specimens were fixed after 24 hours' incubation following exposure. The same results were obtained with both doses (150 e and 270 e). Unlike the 20–25 hour embryos the 6-day old individuals showed no sign of even partial recovery. The heart had ceased to beat and the chick was soft to the touch ; sections showed no mitosis ; the majority of the cells had broken down ; many of the tissues and organs had partially disintegrated and the blood shed into the tissues showed hæmolysis, much of the hæmoglobin having soaked into the amniotic fluid which was clear and bright red in colour.

Summary of Results.—The above experiments showed that in a 6-day embryo fixed *immediately* after exposure mitosis was reduced after 150 e and rare or absent after 270 e. Breaking down cells were numerous in all tissues, and slight extravasation of blood into the tissues was sometimes observed after the larger dose.

After 80 *minutes'* incubation following exposure, mitosis was rare or absent, enormous numbers of breaking down cells were seen in all the tissues, whilst the blood was clotted in the vessels and partly extravasated.

After 24 *hours* mitosis was absent and the majority of the cells had broken down causing a partial disintegration of the tissues.

Table I.

Age of embryos.	Number of embryos used.	Dose (approx.).	Period of incubation following exposure.	Temperature at which incubated after exposure.	Mitosis.	Condition of tissues.	Number of controls.
20–23 hrs.	3	150 e	Fixed immediately	—	Greatly reduced	Unaltered	—
	3	150 e	Fixed after 80 mins.	38° C.	Rare	Slight cell degeneration	1
	4	150 e	Fixed after 24 hrs.	38° C.	Active	Development further advanced; variable amount of abnormal mitosis and cell degeneration	1
	8	150 e	Fixed after 48 hrs.	38° C.	Fairly active	Development further advanced but greatly subnormal; usually much cell degeneration	3
6 days	10	150 e	Fixed immediately	—	Greatly reduced	Unaltered	4
	3	150 e	Fixed after 26 mins.	38° C.	Rare or absent	Breaking down cells appearing	3
	3	150 e	Fixed after 52 mins.	38° C.	Rare or absent	Many breaking down cells; in 2 specimens blood clotted and partly extravasated into tissues	3
	7	150 e	Fixed after 80 mins.	38° C.	Rare or absent	Enormous numbers of breaking down cells; blood clotted and marked extravasation into tissues	5
	4	150 e	Fixed after 5 hrs.	38° C.	Few figures in one specimen	Enormous numbers of breaking down cells; blood clotted and marked extravasation into tissues; partial disintegration of retina and C.N.S.	2
	4	150 e	Fixed after 24 hrs.	38° C.	Absent	Majority of cells broken down; blood clotted and marked extravasation into tissues; almost complete disintegration of retina and C.N.S.	1
20–25 hrs.	4	270 e	Fixed immediately	—	Rare or absent	Unaltered	1
	4	270 e	Fixed after 80 mins.	38° C.	Absent	Numerous degenerating cells	2
	8	270 e	Fixed after 24 hrs.	38° C.	Plentiful	Development further advanced; variable amount of abnormal mitosis and cell degeneration	4
	3	270 e	Fixed after 3 days	38° C.	Present	Cystic or degenerate	—
	3	270 e	Fixed after 4 days	38° C.	Present in one specimen	One specimen normal; remainder degenerate	—
	2	270 e	Fixed after 5 days	38° C.	Present	Cystic	—
	3	270 e	Fixed after 6 days	38° C.	Present in two specimens	Cystic or necrotic	—

6 days	4	270 e	Fixed immediately	—	Rare or absent	Breaking down cells appearing in all tissues; sometimes slight extravasation of blood into tissues	1
	6	270 e	Fixed after 80 mins.	38° C.	Rare or absent	Enormous number of breaking down cells in all tissues; blood clotted and marked extravasation into tissues	2
	2	270 e	Fixed after 80 mins.	0° C.	Absent	Fair number of breaking down cells; tissues otherwise unaltered	—
	11	270 e	Fixed after 24 hrs.	38° C.	Absent	Majority of cells broken down; partial disintegration of tissues; blood clotted and marked extravasation into tissues	3
	2	270 e	Fixed after 24 hrs.	5° C.	Absent	Many breaking down cells; slight extravasation of blood into tissues	—
	2	270 e	Fixed after 24 hrs.	0° C.	Absent	Fair number of breaking down cells; tissues otherwise unaltered	1
	2	270 e	Fixed after 24 hrs. in moist chamber	38° C.	Present	Tissues highly degenerate except for layer beneath dorso-lateral surface in which mitosis occurs	—
17 days	6	270 e	Fixed immediately	38° C.	Rare or absent	Few breaking down cells; tissues otherwise unaltered	2
	5	270 e	Fixed after 80 mins.	38° C.	Absent	Breaking down cells numerous, especially in gut mucosa	—
	7	270 e	Fixed after 24 hrs.	38° C.	Present in one specimen only	With one exception extensive cell degeneration and partial disintegration of tissues	1

C.—*Experiments on 17-day embryos.*

Object of Experiments.—To investigate the effects of radiation on a 17-day embryo as compared with the effects upon a 6-day embryo.

Technique.—The eggs were exposed to a dose of 270 e. The embryos were removed from the shell for macroscopic and histological examination (*a*) immediately after exposure, and (*b*) after exposure and a subsequent incubation period of 80 minutes or 24 hours.

Results.—Embryos removed from the shell *immediately* after exposure showed no macroscopic change. The limbs moved actively, the heart was beating vigorously and the body was normal in colour. Histological examination of the various tissues and organs showed, however, that mitosis had practically disappeared, although except for the presence of a few degenerate cells the tissues were otherwise normal.

Embryos examined after 80 *minutes'* incubation following irradiation were also alive, with one exception, and showed vigorous muscular movements and pulsation of the heart. In the three specimens including the dead individual, the skin was distinctly red. In the histological preparations mitosis was almost absent, and extensive cellular degeneration was observed in the intestine and pancreas and less strikingly in the liver ; the retina, unlike that of the irradiated 6-day embryo, was almost unaffected.

Only one embryo was found alive after 24 *hours'* incubation following irradiation, but this individual showed marked hæmorrhagic changes in the legs and some of the viscera. The dead specimens exhibited varying degrees of redness of the skin, muscles and viscera ; the tissues were extremely soft, the feathers coming away from the skin at the least touch and the brain being a mere pulp. Histological examination of the dead specimens showed absence of mitosis and extensive cell degeneration, the latter being especially marked in the mucosa of the gut and in the retina, both of which had almost completely disintegrated. In sections of the embryo which had been alive at the end of the 24-hour incubation period following irradiation, mitosis was present, although much reduced in amount, and a fairly large number of breaking down cells were seen ; the tissues were otherwise normal.

Summary of Results.—From these experiments it was seen that the effects of X-rays upon 17-day embryos were very similar to, but rather less severe than the effects upon 6-day embryos.

Discussion (Part I).

The experiments described in Part I of this communication showed that the destructive effect of a given dose of X-rays upon the tissues of an embryo was correlated with the age of the embryo. The first question to be decided was why the tissues of irradiated 6-day and (with one exception) 17-day embryos died after 24 hours' incubation, whilst the tissues of 20–25-hour embryos which in some cases had been simultaneously irradiated with 6-day specimens underwent a partial recovery during the 24 hours subsequent incubation.

This disparity in tissue reaction might be explained in two ways: (1) That the tissues of the older embryos were less resistant to the action of the rays than the tissues of the early embryos and that the cells were therefore too seriously affected to permit of their recovery, or (2) that owing to the more complex organisation or larger size of the 6- and 17-day embryos, irradiation produced physiological disturbances which were absent or much less pronounced in the small, simply organised 20-25 hour individuals.

In order to determine if possible which of these two hypotheses was correct, the experiments recorded below in Part II were carried out.

PART II.

The behaviour in vitro *of tissue fragments from irradiated embryos which had been incubated* in ovo *for various periods after irradiation.*

Object of Experiments.—To discover how far the destructive action of irradiation upon the body tissues was due to the direct effect of X-rays upon the individual cells.

Series 1.—Technique. Eggs of 6 days' incubation were exposed to X-rays and received doses of either 150 e or 270 e; essentially the same results were obtained with both doses. After exposure the eggs were returned to the incubator for 80 minutes; the embryos were then removed from the shell and fragments of tissue from the heart, mesonephros or intestine, sclerotic with choroid and skin were removed from the irradiated embryos and explanted *in vitro*.

Results.—The tissue fragments from the irradiated chicks grew readily, showing active outwandering and a return of mitosis (see Table II) and remained in a state of active growth during the 4 days for which they were cultivated. It will be seen from Table II that the mitotic figures were less abundant than in the control cultures of normal unirradiated tissue. This may probably be attributed to the fact that, as described above, a considerable proportion of the cells in an irradiated 6-day embryo were found to be degenerate, or dead and disintegrated after 80 minutes' incubation following irradiation. Thus the number of uninjured cells capable of recovery was smaller per unit volume of tissue in the irradiated than in the normal tissue.

Table II.

Number of embryos used for culture.	Dose (approx.).	Period of incubation following exposure.	Temperature at which incubated after exposure.	Tissue extract.	Average number of mitotic figures per culture.				
					Heart.	Mesonephros.	Intestines.	Sclerotic.	Skin.
8	150 e	80 mins.	38° C.	Normal embryo	11·1	2·5	—	21·8	8·1
3	None (control)	—	38° C.	,,	13·4	3·2	—	33·3	16·5
8	150 e	24 hrs.	38° C.	Normal embryo	No growth	No growth	—	No growth	No growth
2	None (control)	—	38° C.	,,	Normal	Normal	—	Normal	Normal
1	270 e	80 mins.	38° C.	Normal embryo	5	—	9	2·5	3
1	None (control)	—	38° C.	,,	15·5	—	0·5	45	17·5
1	270 e	80 mins.	0° C.	Normal embryo	7·5	—	1·5	11·5	7·5
2	270 e	5 hrs.	38° C.	Normal embryo	No growth	—	Growth in 1 specimen	3·3	3·3
2	270 e	5 hrs.	0° C.	Normal embryo	13·3	—	0·7	15·7	6·3
3	270 e	24 hrs.	38° C.	Normal embryo	No growth	—	No growth	No growth	No growth
2	None (control)	—	38° C.	,,	Normal	—	Normal	Normal	Normal
3	270 e	21–25 hrs.	5° C.	Normal embryo	4·5	—	3·5	6	6
1	None (control)	24–25 hrs.	5° C.	,,	10	—	0	7	1
2	None (normal)	—	38° C.	Embryo irradiated (150 e) and incubated 24 hrs. at 38° C.	13·5	—	2·5	12·5	22·5
2	None (control)	—	38° C.	Normal embryo	8·5	—	1·5	13	16·5
7	270 e	24 hrs. in moist chamber	38° C.	Normal embryo	0 (growth from 1 embryo only)	—	No growth	7·6 (growth from 6 embryos only)	6·5 (growth from 1 embryo only)
2	None (control)	24 hrs. in moist chamber	38° C.	,,	0 (growth from 1 embryo only)	—	3 (growth from 1 embryo only)	6	0 (growth from 1 embryo only)

Series 2.—Technique. The technique was the same as in Series 1 except that the embryos all received a dose of 270 e and were removed from the egg after 5 *hours'* incubation following exposure. Fragments of the heart, intestine, sclerotic with choroid and skin were removed and explanted *in vitro*.

Results.—Only the sclerotic with choroid, the skin and in one specimen the intestine showed growth, and as will be seen from Table II the average number of mitotic figures present in each culture was only 3·3.

Series 3.—Technique. Eggs were irradiated with doses of either 150 e or 270 e and were then returned to the incubator for 21–25 *hours*. Fragments of the heart, intestine or kidney, the sclerotic with choroid and the skin were removed for explantation *in vitro*.

Results.—No sign of growth was obtained in a single culture from any of the 11 embryos used, although the controls grew normally.

Summary of Results.—The foregoing experiments showed :—

1. That a large proportion of the cells of a 6-day embryo irradiated with 150 e or 270 e and then incubated *in ovo* for 80 minutes were alive, and when removed from the body and cultivated *in vitro*, underwent mitosis and remained in an apparently healthy condition for 4 days (1 sub-culture).

2. That in embryos irradiated with 270 e and incubated *in ovo* for 5 hours after exposure a smaller proportion of the cells were capable of continuation of life *in vitro*, and the amount of mitosis was less than in the previous series of experiments.

3. That tissue taken from embryos which had been irradiated with 150 e or 270 e and then incubated *in ovo* for 21–25 hours, showed no trace of growth when explanted *in vitro*.

Discussion (Part II).

The results of the above experiments upon the tissues of 6-day embryos proved that although a certain limited number of the cells were destroyed by the direct action of X-rays, the bulk of the tissues were not killed in this way, but were able to recover from the effects of irradiation when cultivated *in vitro* provided they were removed from the chick within a few hours after exposure. There was no evidence that the cells *qua* cells of a 6-day embryo were more susceptible to the action of X-rays than those of a 20–25-hour embryo.

It was obvious, therefore, that with the exception of the relatively small number of cells destroyed by the direct action of radiation the death of the tissues in the 6-day embryos was the result of some indirect action. It seemed not improbable that the arrest of the circulation owing to the clotting of the blood in the vessels might be the factor chiefly responsible. Assuming this hypothesis to be correct, it remained to determine the precise manner in which the arrest of the circulation could kill the tissues within 24 hours. This might conceivably be effected by the continued metabolic activity of the tissues proceeding in the absence of a supply of fresh material for the needs of the cell or by the accumulation of toxic substances produced by metabolism or liberated by the broken down cells; such substances would probably have been removed from the tissue fragments taken for cultivation *in vitro* by washing in Ringer's solution, and therefore would not have prevented subsequent growth. The experiments recorded in Part III were undertaken in the hope of elucidating this problem.

PART III.

A.—*The effects of low temperatures upon the degenerative changes induced by X-rays in 6-day embryos.*

Object of Experiments.—To show whether the inhibition of metabolism after irradiation would prevent the death of the tissues.

Series 1.—Technique. The eggs after 6 days' incubation were exposed to a dose of 270 e, after which they were placed in cold storage at 0° C. or 5° C., for periods of 80 minutes, 5 hours and 24 hours. When removed from the cold storage the embryos were taken from the shell and fixed and sectioned.

Results.—Irradiated embryos removed from the shell after being kept at 0° C. for 80 *minutes*, 5 *hours*, and 24 *hours* following exposure appeared to the naked eye unaltered or slightly red. Sections presented the histological appearance of irradiated individuals fixed immediately after irradiation, *i.e.*, mitosis was absent, a comparatively small proportion of degenerate cells was seen and the tissues were otherwise unchanged.

The two irradiated embryos fixed after being kept at 5° C. for 24 hours after irradiation showed rather more change, a comparatively large proportion of degenerate cells being seen; they nevertheless presented a striking contrast to individuals fixed after incubation for 24 hours at 38° C. (see p. 15), and it was found that the degenerative changes in the specimens kept at 5° C. for 24 hours were considerably less pronounced than those observed after 80 minutes' incubation at 38° C.

Series 2.—Technique. Four embryos were irradiated simultaneously with a dose of 270 e, after which two were returned to the warm incubator for 5 *hours*, and two were placed in the refrigerator at 0° C. for 5 hours. The embryos were then removed from the egg and fragments of the heart, sclerotic with choroid, intestine and skin were explanted *in vitro*. These experiments were repeated with four irradiated embryos, two of which were incubated for 21–25 *hours* at 38° C., whilst the other two were kept at 5° C. for 21–25 hours. As before, tissue fragments were explanted from all four embryos.

Results.—In the case of the embryos kept in the cold for 5 *hours* fairly good growth was obtained from all the four tissues cultivated. On the other hand, as already described in the case of the embryos incubated at 38° C. for 5 hours no growth took place from the heart in any culture, growth was obtained from the intestine in one embryo only, whilst growth with scanty mitosis was obtained in all cases from the choroid with sclerotic and from the skin. None of the fragments from either embryo incubated for 24 *hours* at 38° C. showed any sign of growth, whilst fair growth with mitosis was obtained from all four tissues in the case of the embryos kept at 5° C. for the same period.

Summary of Results.—The results showed that in 6-day embryos irradiated with 270 e the degenerative changes produced in the tissues by the indirect action of X-rays were greatly delayed, if not completely arrested, by maintaining the irradiated embryo at a low temperature after exposure and thus inhibiting metabolism. Tissue fragments removed from embryos kept at a low temperature for as long as 25 hours after irradiation showed fair growth with mitosis when cultivated *in vitro*.

B.—*The effect upon tissue-growth of extracts of irradiated 6-day embryos incubated at 38° C. for 24 hours after exposure.*

Object of Experiments.—To show whether the death of the tissues was due to the accumulation of toxic substances either produced by metabolism or liberated by the cellular disintegration following irradiation and subsequent incubation.

Experiment 1.—Technique. Two eggs of 6-days' incubation were irradiated with a dose of 270 e. After exposure the embryos were incubated *in ovo* for 21–25 hours at 38° C. and removed from the shell in the usual way. Fragments of the heart, intestine, sclerotic with choroid and skin were removed and explanted *in vitro*. From the remaining tissue of each embryo a saline extract was prepared in the usual way, a reddish translucent liquid being obtained. Fragments of the heart, intestine, sclerotic with choroid and skin from a normal embryo were then cultivated in a medium composed of one drop of the extract made from the irradiated embryos and one drop of plasma.

Results.—When removed from the shell the irradiated embryos were red in colour, the heart had stopped beating, and the amniotic fluid was coloured with hæmoglobin ; the flesh was of very soft consistency. No growth was obtained from any of the cultures made from these specimens, indicating that the cells were dead. On the other hand, the normal tissue fragments cultivated in medium containing saline extract made from irradiated embryos grew as well as the controls for which normal embryonic extract was used, and showed active mitosis.

Experiment 2.—Technique. A 6-day embryo was irradiated *in ovo* with a dose of 150 e and was then incubated for 24 hours. At the end of this period the embryo was removed from the shell and tissue fragments were explanted *in vitro*, whilst from the remains of the chick a saline extract was prepared. Fragments of the heart and sclerotic with choroid of a normal embryo were then explanted *in vitro* into a drop of the extract made from the irradiated embryo, *no* plasma being added.

Results.—The tissue fragments from the irradiated embryo showed no growth *in vitro* but normal embryonic tissue grew very readily in the extract made from the irradiated chick.

Experiment 3.—Technique. A 6-day embryo was irradiated with a dose of 270 e, incubated *in ovo* for 24 hours, and then removed from the shell when it was found to be of soft consistency and red in colour. It was then finely minced and transferred to a centrifuge tube *without* the addition of saline and centrifuged at high speed for about 20 minutes ; a supernatant fluid of a bright red colour was thus procured. Fragments of the heart and sclerotic with choroid from a normal 6-day embryonic chick were cultivated *in vitro* in drops of this fluid *without* the addition of plasma.

Results.—The cultures of normal tissue grew vigorously in the fluid obtained from the irradiated embryo.

Summary of Results.—From the above experiments it was seen that normal tissue would grow readily in the extract of an irradiated 6-day embryo incubated *in ovo* for 21–25 hours following exposure even when such extract was undiluted with saline. These results showed that the death of the tissues of an irradiated 6-day embryo was not due to the formation of stable non-volatile substances.

C.—*The effect of exposure to the air upon tissues of an irradiated 6-day embryo.*

Object of Experiments.—To show whether the death of the tissue could be prevented by permitting gaseous exchange.

Technique.—Ten embryos were irradiated *in ovo* with 270 e ; nine of these embryos were removed aseptically from the shell immediately after exposure, whilst the tenth egg was returned unopened to the incubator (38° C.) for 24 hours. Two of the specimens removed immediately were then partly torn to pieces so as to expose to the air as large a surface as possible whilst the other seven were kept whole. The nine embryos were then incubated at 38° C. in moist chambers under sterile conditions for 24 hours. Two of the seven whole specimens were fixed and sectioned, the other seven embryos together with the irradiated specimen incubated *in ovo* for 24 hours were used for cultivation, fragments of the heart, intestine, sclerotic with choroid and skin being explanted *in vitro*. Two normal unirradiated embryos were removed from the shell and incubated for 24 hours in a moist chamber in a similar way to serve as controls.

Results.—Macroscopic examination showed that the pulsation of the heart had stopped in all 10 embryos; in 2 of the embryos incubated in a moist chamber a certain amount of reddening was already present immediately after exposure, but after the 24 hours' incubation period this had not increased, and the remaining embryos were of normal colour; the embryo incubated *in ovo* showed the usual reddening; the consistency of all the embryos was soft.

It was found that none of the cultures made from the irradiated embryo incubated *in ovo* showed any signs of growth. Cultures made from one of the torn-up individuals also showed no growth whatever. In the case of the other embryos incubated in a moist chamber very fair growth with mitosis was obtained in every case from the cultures of the sclerotic with choroid from both the irradiated and control specimens; a single heart fragment from one of the torn-up irradiated embryos and a single heart culture from one of the controls showed a rather poor scattered outgrowth with no mitosis; growth from the skin was obtained from one irradiated and one control embryo; a single culture of the intestine from one of the control individuals showed slight growth.

Sections of the fixed embryos showed that the greater part of the body consisted of extremely degenerate tissue in which mitosis was absent. Immediately beneath the surface in the dorsal and dorso-lateral regions there was a layer of tissue in which degeneration, though marked, was very much less pronounced; this layer was in most places fairly sharply demarcated from the underlying, highly degenerate tissue described above, and in some areas mitotic figures, usually of a somewhat abnormal type, were not uncommon. The dorsal part of the sclerotic and in one specimen some of the distal cartilages of the limbs appeared almost normal whilst a short piece of the intestine which accidentally had been pulled through the body wall also appeared comparatively healthy and contained mitosis. The entire brain was degenerate, but the necrotic changes were considerably less advanced in the superficial parts than in the tissue lying more remote from the skin.

Summary of Results.—These specimens showed that although tissue cultures from an irradiated 6-day embryo incubated *in ovo* at 38° C. for 24 hours in no case showed the slightest signs of growth, yet tissue cultures of the sclerotic with choroid of similar embryos exposed to the air in a moist chamber and incubated at 38° C. for 24 hours, with the exception of cultures from one embryo, grew readily, showing active outwandering and mitosis.

Discussion (Part III).

From the results of the experiments described in Part III it was evident that the degenerative changes induced in the tissues of 6-day embryos by X-rays were intimately related to cell metabolism, since they were inhibited or greatly retarded when metabolism was arrested by low temperatures. It was also clear that the lethal action of the rays was not due to the formation of stable toxic products. The results of the experiments in which irradiated embryos were incubated for 24 hours while being exposed to the air of a moist chamber instead of *in ovo*, indicated that the death of the cells was due, in part at least, to an absence of gaseous exchange in the tissues of the irradiated chick when incubated in the shell. Such inhibition of gaseous exchange would result from the clotting of the blood in the vessels shortly after irradiation, as described in Part I. On the assumption that the rapid degeneration of the tissues of a 6- or 17-day embryo was due to the inhibition of gaseous exchange owing to the arrest of the circulation, then the contrast between the effect of radiation upon the tissues of the older 6-day and 17-day embryos and the effect upon the younger 20–25-hour individuals in which a circulation was not yet established is easy to understand.

It is unlikely, however, that the inhibition of respiratory exchange is the only destructive effect of radiation since the recovery of the 20–25-hour embryos, as described, was only partial and temporary. It was found, moreover, that in the case of the irradiated 6-day embryos degenerative changes took place much more rapidly than in a normal 6-day individual in which the circulation had been arrested by extirpation of the heart or ligation of the umbilical cord. This problem requires further experimental investigation.

GENERAL SUMMARY.

Part I.

1. *The effect of X-radiation upon 20–25-hour embryos.*

(*a*) Mitosis was very much reduced in embryos fixed *immediately* after exposure.

(*b*) Mitosis was practically absent in embryos fixed after 80 *minutes'* incubation following exposure, and with the heavier doses considerable cellular degeneration was present.

(*c*) Embryos fixed after 24 *hours'* incubation following exposure had undergone a partial recovery as represented by the return of mitosis and an advance in development.

(*d*) After 48 *hours'* incubation following exposure cellular degeneration was usually advanced and normal development was, with one exception, arrested.

2. *The effect of X-radiation upon 6-day embryos.*

(*a*) Embryos fixed *immediately* after exposure showed a striking reduction in the number of mitotic figures.

(*b*) Degenerate cells began to appear in the tissues immediately (larger doses) or (smaller doses) 26 minutes after irradiation.

(*c*) After 80 *minutes'* incubation following exposure the blood was found to be clotted in, and in part extravasated from the vessels, probably as the result of the liberation of ferments by the broken down cells in the vessel walls. Mitosis was almost or completely absent.

(*d*) The tissues of embryos incubated for 24 *hours* after irradiation showed no sign of recovery, and were in a state of partial disintegration.

3. *The effect of X-radiation upon 17-day embryos.*

(*a*) In tissue fragments fixed *immediately* after the exposure of the embryo mitosis was rare or absent.

(*b*) Tissue fragments from irradiated embryos which had been incubated for 80 *minutes* after exposure showed an almost complete absence of mitosis and the presence of degenerate cells, which were especially numerous in the intestine.

(*c*) After 24 *hours'* incubation following irradiation the embryos with one exception were dead and the tissues highly necrotic.

Part II.

1. Tissue fragments removed from an irradiated embryo which had been incubated for 80 *minutes* after exposure underwent at least a partial recovery when cultivated *in vitro* and showed active outwandering and a return of mitosis.

2. The tissues of an embryo which had been incubated *in ovo* for 24 *hours* after exposure were dead and did not grow when explanted *in vitro*.

Part III.

1. The degenerative changes induced by X-rays were almost, if not completely, arrested at 5° C.

2. A concentrated extract of an irradiated embryo which had been incubated *in ovo* for 24 *hours* after exposure had no inhibitory effect upon normal tissue growth *in vitro.*

3. If an irradiated embryo were removed from the shell immediately after exposure and incubated for 24 *hours* in contact with the air in a moist chamber, fragments of the sclerotic with choroid and, in one case, pieces of the skin showed growth and mitosis when explanted *in vitro.*

General Conclusions.

The results of the experiments recorded above indicate that :—

1. The destructive effect of a given dose of X-rays upon the tissues of an embryo is correlated with the age of the embryo irradiated.

2. With the exception of a relatively small number of cells destroyed by the direct action of radiation the death of the tissues in 6-day embryos is due to an indirect action.

3. There is no evidence that the cells, *qua* cells, of a 6-day embryo are more susceptible to the action of X-rays than those of a 20–25-hour embryo.

4. The degenerative changes induced in the tissues of 6-day embryos by X-rays are intimately related to cell metabolism, since they are inhibited or greatly retarded when metabolism is arrested by low temperatures.

5. The lethal action of X-rays is not due to the formation of stable toxic products.

6. The death of the cells in irradiated 6-day embryos is due, in part at least, to the absence of gaseous exchange in the tissues of the chicks when incubated in the shell; such an inhibition of gaseous exchange would result from the clotting of the blood in the vessels shortly after irradiation.

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The Dynamics of "Sprint" Running.

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(Received May 28, 1927.)

(From the Baker Laboratory of Chemistry, Cornell University, Ithaca, New York.)

CONTENTS.

		Page
1.	The greatest allowable speed of movement	29
2.	The "viscosity" of human muscles	30
3.	The equation of motion of a runner exerting a maximal effort	33
4.	The electrical timing of runners	35
5.	The theory of running uphill	41
6.	Summary	41

The Greatest Allowable Speed of Movement.—Consider a series of "similar animals" of different size and let the linear dimensions of any individual of the series be chacterised by the length l. Let M be the mass of one of its limbs, and v the greatest velocity it can attain in that limb. Then the momentum of the limb is proportional to Mv. Now this momentum is created at the beginning of a movement, or destroyed at the end of a movement, by a force F acting for a time t; and we may write, neglecting constants,

$$Mv = Ft.$$

Now t, the time occupied in producing or checking a movement, is proportional to l/v, while M is proportional to l^3, so that, again neglecting constants,

$$l^3v = Fl/v. \qquad \text{Hence} \quad v^2 = F/l^2.$$

Now the strength of the structures of an animal is proportional to the cross-sections of bones, muscles, tendons, joints; if the force exerted in starting or

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[*] Work done during the tenure of a non-resident lectureship in Chemistry at Cornell University.

stopping each movement of a limb be too great, breakage in these structures will occur. The maximum force F_0, therefore, which the animal dare exert is proportional to l^2; hence the maximum velocity v_0 which it can develop, or check, in its parts, without danger of breakage, being from above proportional to F/l^2, is constant and independent of the dimensions of the animal. In other words, similar animals built of similar materials, should, whatever their size, have the same maximum linear velocity of movement. " Similar " animals are, of course, a mathematical abstraction; the racehorse and the greyhound, however, are built to the same general pattern, and they have roughly the same maximum speed; the cat is not so very much slower than the tiger; even the kangaroo and the grasshopper would not differ very widely in their achievements at the standing broad-jump.

The reason why any animal, including man, is not able to move faster, however great an advantage to him it might be, is that if he did so he would tear his tendons, break his bones, " pull " his muscles, strain his joints. Indeed one of the problems of an athletic coach is to save his best short-distance runners from doing these very things. The first-rate " sprinter " has a much smaller margin of safety than more normal men.

The " automatic brake " or " governor " which prevents too rapid a movement is inherent in the muscle fibres, and is not produced by reflex activity of the nervous system, as is, for example, the reciprocal inhibition of antagonist muscles. General reasoning tells us that such a regulator must be present if an animal is to survive, and it is found to be a universal and essential part of the muscular machine. Its nature has been widely investigated of late, under the general title of the " viscous-elastic " properties of muscle (1)(2).

The Viscosity of Human Muscles.—It notoriously requires more energy to walk or run a given distance fast than slow. If a muscle fibre shortens quickly it does less work than if it shortens slowly, consequently if the same external work has to be done more muscle fibres have to be excited. The arrangement provided to prevent us from moving too fast is the same as makes a higher speed more wasteful.

The work done by a human muscle in shortening can be expressed as a simple function of the speed (3)(4). Let the work done in a maximal effort in an infinitely slow " reversible " shortening be W_0; it is assumed that all the fibres of the muscle are innervated. If the same effort be made, but the shortening be conducted at speed v, the work done W will obey the relation

$$W = W_0 (1 - v/c)$$

where c is the greatest possible speed of shortening of the unloaded muscle. When a muscle shortens it changes its shape; its molecules or parts have to flow into a new configuration; the change is hindered by the hysteresis of the molecular system of the muscle substance, by what we have called its "viscosity," and experiment has shown that, with human arm muscles, the above equation is sufficiently accurately obeyed.

In all the experimental work performed on the subject the contractions employed have been maximal. The only reason for this is a technical one, namely, that the maximal is the only constant muscular effort which it is possible to reproduce exactly, whether in isolated muscles, in animals, or in men, by "constant muscular effort" being implied the response of a constant number of muscle fibres. There can, however, be no doubt that were we able to cause any given fraction $1/p$ of the fibres to contract actively, then the same equation would apply with W_0 merely altered to W_0/p. It was imagined, early in the investigation of these effects (3, p. 37), that the viscosity involved was that of the resting muscle fibre, so that if a smaller number of fibres were involved in a contraction the maximal work would be less, but the viscosity would remain the same; in other words, the active fibres would have to overcome not only their own viscosity but also that of their neighbours. In that case the equation would be

$$W = W_0/p - W_0 v/c,$$

whereas, in fact, it is

$$W = W_0/p\,(1 - v/c).$$

The reason for adopting the second equation, rather than the first, for a submaximal contraction, is that the viscosity of an active fibre is so much greater than that of an inactive one that the latter, practically speaking, can be neglected (see Gasser and Hill (5, p. 417)).

Let us imagine that we have to lift a constant load, or to overcome a constant resistance, at a series of different speeds. At a low speed a small number of muscle fibres will be sufficient to undertake the task, since, shortening slowly, they will all do a large fraction of their maximal work. At a higher speed a large number of muscle fibres will be required, since each, shortening more quickly, will do less work. At some high speed all the fibres will be required. Let the work to be done be W and let the "reversible" work of each fibre be w_0. Let n fibres be required to do the work at speed v. Then

$$W = n w_0\,(1 - v/c)$$

$$n = \frac{W}{w_0\,(1 - v/c)}.$$

The number n of fibres needed to do the work at the required speed is a measure of the physiological effort made by the muscle, and we see that as v becomes larger the number of fibres continues to increase, until finally all the fibres in the muscle are employed. Thus a task W which is easy when carried out slowly may become exhausting when carried out rapidly, for a greater number of fibres will be required, which will result in greater chemical activity, greater oxygen requirement, and greater lactic acid production.

In the case of relatively slow movements, such as rowing, another factor is involved, namely, the energy expended by a muscle in maintaining its contraction. If the contraction be too slow this may more than counterbalance the advantage of using less fibres. Let H be the total energy given out by a muscle stimulated isometrically at its resting unloaded length for time t. Then approximately

$$\mathrm{H} = 2nw_0\,(1 + bt)$$

where n and w_0 have the same meaning as before, and b is a constant depending on the characteristics of the muscle. H is known to be affected, to some degree, by allowing the muscle to do work (Fenn (6) (7)); hence, strictly speaking the above formula cannot be applied to such a case. As an approximation, however, we may apply it, and so we find that the mechanical efficiency is

$$\mathrm{E} = \frac{nw_0\,(1 - v/c)}{2nw_0\,(1 + bt)}.$$

Now approximately v is proportional to $1/t$, if the contraction ends with the movement; so putting $v/c = k/t$ we finally have

$$\mathrm{E} = \frac{1 - k/t}{2\,(1 + bt)}.$$

This expression has a maximum for a certain time $t = t_1$; thus there is a "most efficient" speed, at which any given piece of external work can be accomplished with the least expenditure of energy. The work W being given, n_1 the "optimum" number of fibres required for it can be calculated,

$$n_1 = \frac{\mathrm{W}/w_0}{1 - k/t_1}.$$

It should be noted that here we are concerned with a given amount of external work W in the strict sense, not with such a task as walking or running a given distance. In such forms of exercise as running or walking on the flat, where there is no external work done, apart from the small amount involved in overcoming air resistance, and where there is no "most efficient" speed, the above argument fails. The whole of the mechanical energy liberated is used in overcoming the frictional resistance of the body itself, particularly the "viscosity" of the muscles. Work has to be done in producing and destroying kinetic energy in the alternating movements of the limbs. This kinetic energy is proportional to v^2, so that in running at speed v, mechanical energy has to be expended per yard covered proportional to v^2. This must be derived from the work W in the equation

$$W = nw_0 (1 - v/c),$$

so that writing W as av^2, where a is a constant, the physiological effort, per yard covered, is given by

$$nw_0 = \frac{av^2}{1 - v/c}.$$

Now in running the movements are so rapid that no appreciable amount of energy is used in maintaining contraction in muscles ; it is all used in setting up contraction. Thus the total energy requirement per yard is proportional to the physiological effort nw_0, and therefore to

$$\frac{v^2}{1 - v/c},$$

while the total energy requirement per second is proportional to

$$\frac{v^3}{1 - v/c},$$

since the number of yards covered per second is proportional to v.

We see therefore that the oxygen requirement per second of running should vary more rapidly than the cube of the speed, and this has actually been found by Sargent (8, p. 17) who states that it rises approximately as the 3·8th power of the speed.

The above argument is only very general and no exact results could be expected from it ; changes of stride and style will undoubtedly occur with changing speed : it shows, however, without going into details, why so much more energy is expended in a more rapid run or walk.

The Equation of Motion of a Runner exerting a Maximal Effort.—Let M be the mass of a runner and let us assume that he can exert a maximum force F in propelling himself horizontally on a track. Since, in general, F will be proportional to his weight, we may write

$$F = f M g,$$

where f is a constant of no dimensions, characteristic of the build, strength, skill and fitness of the subject. It will be found usually to vary between 0·5 and 1·0. If there were no resistance inherent in the muscles and limbs of the runner the acceleration would remain large and he would rapidly reach an enormous velocity. Fatigue, of course, soon comes on, but not so soon as to prevent the attainment of a very high speed. Actually in a trained man fatigue has no effect up to 70 yards at top speed, and a maximum velocity is practically attained in about 50 yards. The resistance which rapidly brings his velocity to its constant "limiting" value is that due to the "viscosity" of his muscles.

The force inherent in a human muscle which resists its rapid shortening is proportional to the speed of its movement; it will clearly, other things being equal, be proportional to the size of the muscle, and therefore for similar individuals to the mass of the body. Thus the "viscous" resistance may be taken conveniently as Mv/a, where a is a constant and v is the velocity. This is true only for maximal efforts, since for sub-maximal ones the number of muscle fibres involved is arbitrary, and the frictional resistance is mainly confined to the active muscle fibres. The quantity a has the dimensions of time.

We are now in a position to write down the equation of motion of a subject, up to the stage when fatigue begins to come in. Let y be the distance run in time t, the subject being assumed to be exerting himself to the utmost of his powers throughout. The equation is,

$$M\frac{d^2y}{dt^2} = f\,Mg - \frac{M}{a}\frac{dy}{dt},$$

or dividing by M,

$$\frac{d^2y}{dt^2} = f\,g - \frac{1}{a}\frac{dy}{dt}.$$

This equation can be integrated,

$$y = fga\left[t - a\left(1 - e^{-\frac{t}{a}}\right)\right],$$

and the velocity at any time can be written,

$$\frac{dy}{dt} = fga\left(1 - e^{-\frac{t}{a}}\right).$$

We see that the velocity attains its limiting value, fga, along an exponential curve, and once this limiting value has been practically attained the distance covered increases uniformly with the time: it becomes $y = fga\,(t - a)$. Thus the total time-lag due to inertia acting up to the moment when the maximum velocity has been attained, is easily seen to be a; so that if our subject were able instantaneously to start up with his full velocity fga he would run any distance of 50 yards or more in a seconds less than he does in actual fact.

We have neglected one factor here: the absolute latent period of the subject's movements. It requires a certain time for the order "Go" to be signalled to his brain, and to be sent back to his muscles, and for these to develop their maximum force in response. Let this latent period be b. b is very small in a trained athlete, but may be much more in others. The total lag in starting is really $(a + b)$ seconds, and by t in the above equations we must understand the time measured from the moment when the muscles have developed their response to the signal "Go."

The Electrical Timing of Runners.—It seemed desirable to test this reasoning and these formulæ by actual experiments on athletes. The principle adopted was to time trained runners, exerting their maximum effort, over a series of distances along a horizontal track.

If a vertical plane coil of wire arranged parallel to a track be connected with a galvanometer, and a runner carrying a magnet approach it, the magnet will induce a current in the coil which can be recorded by the galvanometer. The induction from the magnet increases up to the moment when the magnet lies in the "meridian" of the coil, and then diminishes again as the runner moves on. Thus the current induced changes sign as the runner passes through the meridian of the coil.

With a very rapidly moving galvanometer the record would be a diphasic one. With an ordinary moving-coil galvanometer, however, which cannot follow such rapid currents the record appears as a single wave (see fig. 1) with a sharp peak. The more rapid the galvanometer the less lag there will be in this peak. In the present experiments the instrument employed has been a Moll moving-coil instrument of complete period 0·8 second, made by Messrs. Kipp en Zonen, of Delft, Holland. The top of the wave represents the change over from a positive to a negative induced current, and therefore (with a certain small lag—see below) the moment at which the magnet passes the meridian of the coil. The narrower the coil, and the closer the magnet comes to it, the sharper will be the peak of the wave recorded by the galvanometer.

The coils employed were about 60 cm. high and about 40 cm. wide. They were placed parallel to the track and at intervals along it. In all experiments it is desirable to have a number of them near the start, in order to determine the initial acceleration of the runner; and in most cases 10 coils have been employed at distances from the start of 1, 3, 6, 10, 15, 20, 30, 40, 50 and 60 yards. In other cases 15 coils have been used for studying the onset of fatigue, and these have been placed at 1, 3, 6, 10, 15, 20, 40, 60, 80, 100, 120, 140, 160, 180 and 200 yards. The coils were mounted on stands so that their centres were about chest high. Each of them consisted of 200 turns of insulated copper wire,

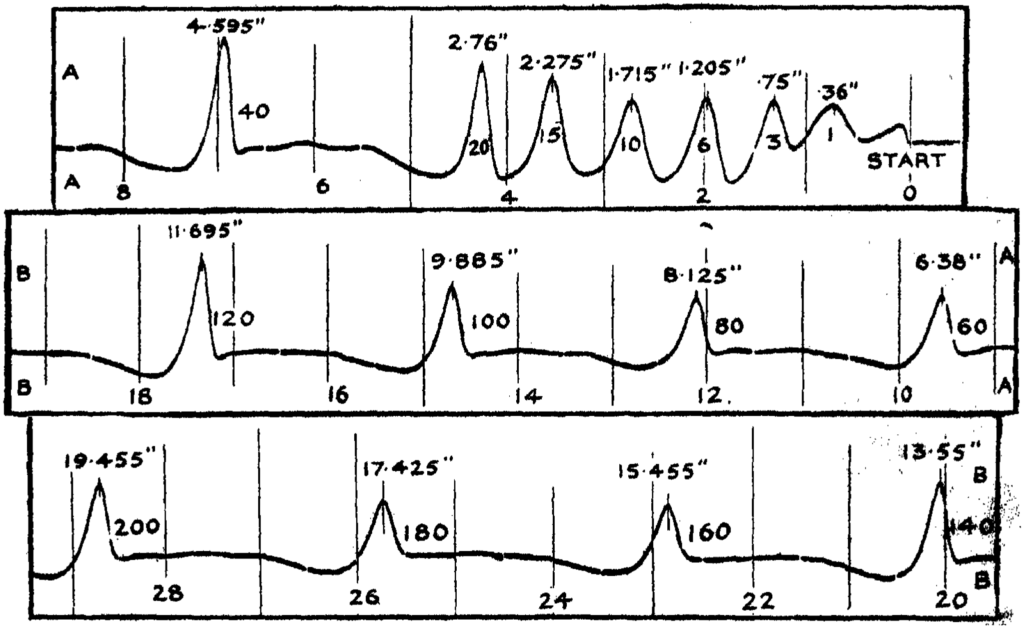

Fig. 1.—Record on H.A.R. over 200 yards. Cut into three portions at AA and BB. Read from right to left and from top to bottom. Distances are given in yards by the side of or beneath the several waves. The cross-lines mark time in units of 0·683 second. The peaks of the waves are marked in for ease of measurement. The subject was started by a pistol, and the starting signal was made by an observer standing near the starter.

0·016-inch diameter, and they were all connected in series with the galvanometer by flexible wire.

The recording arrangements were as follows:—A beam of light from a lamp passed through a narrow slit, to give a vertical line, and was reflected from the mirror of the galvanometer into a camera. There it was concentrated by a cylindrical lens to a spot, which traced out a line on moving bromide paper. The camera was of the kind commonly employed with the string galvanometer (electrocardiograph) and was driven by an electric motor. It was not necessary to darken the room. The paper after exposure dropped through into a box below, where it could be cut off, and then taken to the dark room to be developed. A time marker (metronome) cut off the light momentarily every ½ second (about) and so left a narrow gap in the record by which the latter could be measured up to the nearest 1/200 second. The cross lines shown in the record (fig. 1) were ruled in afterwards, through every alternate gap, to facilitate measurement.

The magnet employed was one or more hack-saw blades, with teeth ground off, and magnetised in a solenoid. It was tied round the chest, or the waist, of the subject with a string or tape. The runner should keep within 1 or 1½ yards of the coils to insure a good sharp record.

To record the start, an electric switch is opened by the starter at the same moment as the signal " Go " is given, or else by an observer at the moment the pistol is fired. This breaks the primary circuit in a transformer, and a current is induced in the secondary. To make the record smaller and more sudden this induced current is put through a second transformer, and the second induced current runs through the coil-galvanometer circuit. The upstroke of the starting signal is extremely sudden and can be read to 0·002 second if desired. If it were required to make the upstroke automatically simultaneous with the order to start an electric switch could be included in the pistol.

Lag.—To determine the lag with the particular galvanometer used two coils were erected about 6 yards apart on a track and the middle point between them carefully marked. A subject carrying a magnet ran at a constant speed of about 7 yards a second along the track, and the record of his passing the two coils was made by the galvanometer. At the moment of his passing the middle point an observer pressed the starting switch and made a sharp up-stroke on the record. The lag in this up-stroke itself is negligible, while the observer, who could see the runner approaching, should have had on the average no lag in recording the moment of his passage. In 30 such observations the signal appeared to occur in the mean 0·127 second earlier than the moment half way between the two coil-records. The probable error of this mean value was small—only about 0·004 second. We have assumed, therefore, that there is a lag of 0·13 second in each observed time (except the start) and subtracted this from all the times. Strictly speaking this correction should apply only to the case of the particular speed of passing the coils at which it was determined. At lower and higher speeds there is probably some small error still remaining. This may have caused a very slight distortion of the shape of the distance-time curves recorded. The lag could only be completely eliminated by employing a more rapid galvanometer.

The consistency with which records can be reproduced by a man in training is remarkable. A subject ran four times in the same afternoon, at intervals of a quarter of an hour, with results given in the following table. (Starting signal simultaneous with " Go.")

Distance, yards :		1	3	6	10	15	20	30	40	50	60
Time, seconds :	(1)	0·54	0·96	1·45	1·99	2·59	3·17	4·25	5·30	6·38	7·43
	(2)	0·50	0·94	1·43	1·97	2·59	3·15	4·23	5·29	6·38	7·45
	(3)	0·49	0·91	1·41	1·95	2·58	3·14	4·23	5·30	6·40	7·47
	(4)	0·54	0·94	1·43	1·97	2·57	3·14	4·22	5·28	6·35	7·37
	Mean	0·52	0·94	1·43	1·97	2·59	3·15	4·23	5·29	6·38	7·43

The average difference between the mean and the observed time for any distance is only 0·014 second. The same consistency was obtained in other cases.

The chief *external* force to be overcome in running on the flat is that due to the resistance of the air. At very low speeds this resistance varies as the relative velocity of object and air; at such speeds as we are concerned with in running, and up to 600 or 700 feet per second, it varies as the square of the relative velocity. Thus a head-wind of 10 miles per hour against a runner going 20 miles per hour will increase the resistance 2¼ times. Now it is well known to runners that a head-wind has a slowing effect—a wind of 10 miles per hour might be expected to increase the time in 100 yards by a few tenths of a second. Conversely a following wind is an aid. We may calculate that a wind of 10 miles per hour behind a runner, going 20 miles per hour, will reduce the air resistance to ¼, and it is found in practice to diminish the time by a few tenths of a second. The effects, however, which are produced by wind are so relatively small—though of course important enough when it comes to record breaking—that no large part of the resistance which the muscles have to overcome can be attributed to the air; a 2 or 3 per cent. decrease in velocity can mean but a very small increase in total resistance. The only other external resistance is that caused by unevennesses in the ground, and this must be negligible on a good track.

Thus, the main part of the resistance encountered by a man running on the flat must be due to frictional forces inside the body itself. These frictional forces, due to what we have called "viscosity" in the active muscle fibres, vary directly as the speed of movement, and so fall into the equation used above to describe the motion of a runner. Any correction to this equation, on account of other forces not varying in this way, will be small and, so long as we are dealing with a first approximation only, can safely be neglected.

It is possible, as a matter of fact, to fit the equation to the observations with a high degree of accuracy. Taking fga as 9·43 (in yard-second units) and a as 1·09, the distances calculated for the observed mean times in the above table compare with the observed distances as follows:—

Observed	1	3	6	10	15	20	30	40	50	60
Calculated	0·97	2·99	6·05	10·07	15·20	20·10	29·9	39·8	50·0	59·9

The average difference between observed and calculated distance is 0·086 yard, that is about 3 inches, and the coils themselves are not placed much more accurately than that. No better agreement could be expected. The observations have been fitted by the equation in this way in dozens of experiments, and the accuracy in the above case is not in the least exceptional.

It is obvious that in a short race we may regard an athlete as being propelled by a constant force, and resisted by a force proportional to his speed. Thus, like a raindrop falling through the air, he rapidly attains a certain limiting speed, and this speed remains constant until fatigue causes it to fall off. The values of *fga* and *a* are determined by fitting the equations to the observations. The value of *fg* can be calculated from *fga* and *a* by division. On dividing further by *g* (10·73 in yard-second units) we obtain the value of the force exerted in terms of the weight of the man.

In the above case the propelling force was 0·806 of the runner's body-weight. The highest value hitherto observed is 0·92, the lowest 0·51. We see therefore that the motion of a runner making a maximal effort can be described in terms of two quantities: (i) the propelling force, which is constant, and (ii) a resistance proportional to his speed at the moment considered.

From the propelling force the mechanical work performed can be calculated. Let *l* be the horizontal distance run, then the work will be *fMgl*. Expressing the results in ordinary engineering units, if M be the runner's mass in pounds and *l* the distance run in feet, the work done will be *fMgl* foot-poundals, or *fMl* foot-pounds. If *f* be 0·85, a value typical of a good short-distance runner, and *l* be 300 (100 yards) the work done by the subject will be 255 M foot-pounds, that is enough to raise his body 255 feet vertically upwards. If M be 160 pounds, this becomes 40,800 foot-pounds; if, further, the time for the 100 yards be 10 seconds, the work done will be 4,080 foot-pounds a second. One horse-power is 550 foot-pounds a second, so that such a runner will be developing about 7·4 horse-power of mechanical energy while running at the speed considered. It is obvious why fatigue comes on so rapidly during exercise of this character.

In fitting the equation to observations made in this way the following method has been found convenient. Without some such procedure much time will be wasted.

The maximum speed is determined, either by plotting the points and laying off the line of greatest slope, or by direct calculation from the intervals. This gives the value of *fga*. A value of *a* is then selected in the following table, and the numbers opposite it are all multiplied by the value of *fga*. These are then plotted, and if *a* be correct the resulting curve should coincide with that drawn through the observations, or rather should run parallel with it, as there is generally a small allowance to be made for slow or premature starting. Usually there is a divergence of the two curves and another value of *a* must be tried. A few trials, however, are always sufficient to make a good fit.

Table.—Values of $t - a\left(1 - e^{-\frac{t}{a}}\right)$.

Time *t* seconds.

a.	$e^{-(1/a)}$.	0·5	1·0	1·5	2·0	3·0	5·0	7·0
0·830	0·30	0·125	0·419	0·808	1·245	2·194	4·173	6·170
0·853	0·31	0·122	0·412	0·795	1·230	2·173	4·150	6·147
0·877	0·32	0·119	0·404	0·783	1·214	2·152	4·126	6·123
0·902	0·33	0·116	0·396	0·770	1·197	2·131	4·102	6·098
0·927	0·34	0·113	0·388	0·757	1·180	2·110	4·078	6·074
0·952	0·35	0·110	0·381	0·745	1·165	2·089	4·053	6·049
0·979	0·36	0·108	0·374	0·733	1·150	2·068	4·028	6·024
1·005	0·37	0·106	0·367	0·721	1·133	2·046	4·002	5·996
1·033	0·38	0·104	0·359	0·709	1·116	2·023	3·975	5·968
1·061	0·39	0·102	0·353	0·698	1·100	2·002	3·949	5·940
1·090	0·40	0·100	0·346	0·685	1·085	1·980	3·921	5·911
1·121	0·41	0·097	0·339	0·673	1·068	1·957	3·892	5·882
1·152	0·42	0·095	0·332	0·661	1·051	1·933	3·863	5·851
1·183	0·43	0·093	0·325	0·649	1·035	1·910	3·836	5·820
1·217	0·44	0·091	0·319	0·637	1·018	1·887	3·806	5·789
1·252	0·45	0·088	0·312	0·625	1·001	1·862	3·773	5·752
1·287	0·46	0·086	0·305	0·614	0·985	1·837	3·740	5·720
1·325	0·47	0·084	0·298	0·602	0·969	1·814	3·707	5·683
1·362	0·48	0·082	0·291	0·590	0·952	1·790	3·674	5·649
1·401	0·49	0·080	0·286	0·579	0·935	1·765	3·640	5·609
1·442	0·50	0·078	0·280	0·569	0·918	1·739	3·606	5·570

The following values have been obtained for the constants in a number of subjects :—

Subject.	Max. speed *fga*	$e^{-(1/a)}$.	*f*
H.A.R. (1)	11·46	0·46	0·83
„ (2)	11·23	0·46	0·815
10 men averaged together	10·06	0·42	0·815
Fi (mean of 4)	9·43	0·40	0·805
S (mean of 4)	9·43	0·40	0·805
O (mean of 4)	9·37	0·35	0·92
G (mean of 4)	9·43	0·37	0·87
Y (1)	9·86	0·41	0·82
Y (2)	10·0	0·40	0·855
S	8·24	0·31	0·90
P (mean of 3)	7·63	0·38	0·69
F (mean of 5)	7·30	0·38	0·66
H (mean of 10)	8·41	0·46	0·61

Of these H.A.R. is a first-rate short distance runner ; the equation fitted his records very exactly. All the others, down to and including Y, are good short distance runners. S is an oarsman ; P and F are not athletes ; H, now aged 40, used to be a middle-distance runner. The results show that H.A.R. is superior to the other short distance runners in one respect only, namely, in the smallness of his " viscosity," that is in the largeness of $e^{-\frac{1}{a}}$. He is superior, however, to H in a different respect, namely, in the size of his propelling force, that is in f. The characteristics of the different individuals, for the days and under the conditions of the observations, are completely described by their respective constants.

The Theory of Running Up Hill.—The above considerations may be applied to the case of a man running up hill. In this case the external work is greater (it is almost negligible in running on the flat) and therefore the speed must be less. Let R be the external resistance, then the equation becomes

$$M\frac{d^2y}{dt^2} = (fMg - R) - \frac{M}{a}\frac{dy}{dt}.$$

It is easy to see that the maximum velocity, instead of being fga, will be,

$$a\left(fg - \frac{R}{M}\right).$$

Suppose the subject to run up a slope of 1 in n. n must be fairly large, or otherwise the style of running will be changed, probably a slope of 1 in 10 would be the greatest practicable, possibly even this would be too high. The resistance R is now Mg/n, so that the maximum velocity becomes

$$(f - 1/n)\,ga.$$

If f were 0·6 and n were 10, this would mean that the velocity would be reduced in the ratio of 6 : 5, in other words a subject capable of doing 100 yards in 12½ seconds on the flat would take 15 seconds up such a slope. It would be interesting to carry out comparative experiments in timing the same subject by the methods described above on tracks of different slopes. To obtain a greater effect the subject should be one with a small value of f; probably a long-distance runner would prove most suitable.

Summary.

1. It is shown, by an application of the theory of dimensions, that the speed of an animal such as man is limited by the inertial stresses to which his structures are subjected during movement.
2. The "viscosity" of the muscles is the chief factor regulating the speed of movement.
3. For experimental work on muscle "viscosity" it is necessary to employ maximal contractions. A theoretical discussion is given of the case of submaximal contractions.
4. The high physiological cost of rapid movement, as in running and walking fast, is explained.

5. On the assumption that the force exerted by a runner is used mainly in overcoming the " viscosity " of his own muscles, the equation of motion of a runner starting from rest and exerting a maximal effort is given.

6. An electrical method of timing a runner is described: by means of it the complete distance-time relation over any distance can be determined.

7. It is shown that a runner exerting a maximal effort propels himself with a constant force and is retarded by a resistance proportional to his speed.

8. The " constants " of a given runner, namely, propelling force and coefficient of viscous resistance, can be determined with very fair accuracy. They vary widely in different healthy individuals.

9. The work done in running, against the viscous resistance of the muscles, can be determined.

10. The theory has been extended to the case of running up hill.

Our sincere thanks are due to Prof. L. M. Dennis and the staff of the Baker Chemical Laboratory for their hospitality, and for the continual help we have received during the conduct of this work.

Our most grateful thanks are due also to the athletic authorities at Cornell University, to Mr. R. Berry and Mr. J. F. Moakley; and particularly to the runners themselves, whose friendly co-operation made the experiments possible.

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The Energy Used in "Sprint" Running.

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(Received May 28, 1927.)

(From the Baker Laboratory of Chemistry, Cornell University, Ithaca, New York.)

CONTENTS.

		Page.
1.	The mechanical efficiency of the body in running	43
2.	The onset of fatigue	47
3.	Summary	50

The large amount of energy used in muscular exercise of extreme severity has been demonstrated by recent work on the oxygen requirement of such exercise. Sargent (1, p. 16), for example, has found an oxygen requirement of nearly 30 litres per minute in a subject running 120 yards at an average speed of 9·23 yards per second, which is about eight times his maximum oxygen intake. Our previous paper will have explained how this great expenditure of energy occurs; it is not used mainly in overcoming any resistance external to the body, but rather the "viscosity" of the muscles of the subject himself. The mechanical work done can be calculated by the methods outlined in that paper, and it is of interest to compare it with the oxygen used in recovering from the effort made, which is a measure of the total energy expended.

The Mechanical Efficiency of the Body in Running.—For the purpose of these experiments the subject is first caused to rest for 45 minutes, and a measurement is made of his resting oxygen consumption. Then, without any other exercise, he is required to run 60, 100, or 150 yards at top speed, to pull up as rapidly as possible, and to collect his expired air for 30, 40 or 50 minutes, depending on the time needed for complete recovery. It should be noted that at least 30 minutes is required, at any rate in the subjects we have studied. Finally, a second measurement of his resting oxygen consumption is made, which, for a satisfactory experiment, should be the same as the first. The excess oxygen due to the exercise can then be determined. For the greatest accuracy the subject should not allow any of his expired air to escape, after the moment he starts to run: it should all be collected. This means that he must hold his breath while running, pulling up and fixing the mouthpiece; this is easy in a

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* Work done during the tenure of a non-resident lectureship in Chemistry at Cornell University.

60-yards run, possible in a 100-yards run, very difficult in a 150-yards run. Even, however, if two breaths (say 6 litres) of expired air be lost in the longer distance, this will not be more than 2 per cent. of the excess ventilation of the lungs in 30 minutes of recovery, and so will produce only a small error ; and in the shorter distances this error can be avoided completely. Thus, provided that no serious amount of expired air be lost, the exercise can be taken without any impedimenta of mouthpiece and bag, and in the natural way, on an ordinary running track* : the whole of the oxygen required is taken in during recovery.

A more serious error, and one not so easily avoided, is that due to the pull-up. A man running at top speed can stop in about 10 yards, and the effort made in stopping is of a less muscular character than that involved in running and so should involve less loss of energy. In the experiments made on three different subjects in this connection we have assumed that the effort made in stopping is equivalent to that of running for another 5 yards ; it cannot be far from this, for 10 yards of stopping is certainly not as energetic as 10 yards of running at maximum speed. The correction for stopping is reduced in importance by taking a longer distance, such as 150 yards, where it represents a smaller part of the whole.

Another error, also comparatively unimportant, is that due to the onset of fatigue. In 60 or 70 yards the maximum speed, once reached, is maintained to the end. In 150 yards it falls off by about 5 per cent. If we assume that the propelling force fMg diminishes in the same ratio as the speed, we can make an appropriate correction. This again may not be exact, since the " viscosity " also may change with fatigue, but in 60 and 100 yards the correction is negligible, and in 150 yards comparatively small, so that no great error can result.

We have therefore all the data for a comparison of the mechanical output of the muscles with the energy liberated as calculated from the oxygen. With regard to the calculation, it would seem certain (as we shall show in a later paper) that in recovery from exercise such as this the only substance oxidised is carbohydrate, so we may safely take 1 litre of oxygen as being the equivalent of 15,860 foot-pounds of work.†

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* Actually we employed a straight concrete path.

† Distances being measured in yards and feet on a track it has been more convenient in the present experiments to work with English units. Glycogen (Slater (2)) has a heat of combustion, in solution, of 3836 calories per gram, hence 22·4 litres of oxygen, used in burning 30 grams of glycogen, give an amount of heat 30 × 3836 calories, so that

$$\begin{aligned}1 \text{ litre } O_2 &\equiv 5140 \text{ calories}\\ &\equiv 2190 \text{ kilogram-metres}\\ &\equiv 15860 \text{ foot-pounds.}\end{aligned}$$

Typical results in such an experiment are as follows:—

> Subject 161 pounds; distance 60 yards; oxygen used in recovery 3·81 litres; pull-up taken as equivalent to 5 yards run; propelling force, calculated from distance-time record, 0·70 of body weight; work calculated = 161 × 0·7 × 65 × 3 = 22000 foot-pounds; energy used = 3·81 × 15860 = 60300 foot-pounds; efficiency = 36·5 per cent.

In the following Table, summarised, are all the experiments made in this connection, neglecting none. It is seen that the mechanical work done is, on the average, about 38 per cent. of the total energy used. The ratio is not quite constant, but the complexity of the lengthy observations involved and the many possibilities of error, both in the oxygen measurements and in the interpretation of the timing data, are sufficient to warrant a belief that the "mechanical efficiency" of sprint running is really constant at about 38 per cent.

Table I.

Subject A	Distance 60 yards	Efficiency 36·5 per cent.
	,, 100 ,,	,, 33·3 ,,
	,, 100 ,,	,, 29·8 ,,
	,, 150 ,,	,, 36·7 ,,
	,, 150 ,,	,, 37·7 ,,
	Mean for this subject	35·0
Subject B	Distance 60 yards in all experiments.	
		Efficiency 40·1 per cent.
		,, 41·6 ,,
		,, 36·9 ,,
		,, 42·2 ,,
		,, 44·5 ,,
		Mean 41·1 ,,
Subject C	Distance 60 yards in all experiments.	
		Efficiency 39·6 per cent.
		,, 36·1 ,,
		,, 34·9 ,,
		Mean 36·9 ,,

Mean of all experiments made: Efficiency 37·7 per cent.

The value we have arrived at is of great theoretical interest. In muscle activity, at least in frogs, it is known that of 100 units of energy liberated in the complete cycle 40 occur anaërobically in the initial process, 60 in recovery. Actually 100 units of energy are set free by oxidation in recovery, of which 60 appear as heat, 40 go into the latent form of chemical energy in effecting the restoration of the muscle. Thus, if the whole of the initial energy were liberated as mechanical work, the efficiency would be 40 per cent. Now in the processes of running a great amount of effort is wasted: the arms are flung about, the legs are pulled violently forward as well as forced back in propelling the body, even the muscles of the face and neck are involved.

It seems inconceivable that the muscles involved in pure propulsion should not have an efficiency notably above that of the body as a whole. If they have, that efficiency must be well above 40 per cent.; in other words, the mechanical energy they can give out is greater than the whole of the initial energy. There is, as a matter of fact, no theoretical difficulty in this: many chemical reactions are known in which the free energy, the maximum work, is greater than the total energy. Furthermore, experiments on the isolated muscle readily demonstrate that the area of the tension-length curve (representing the theoretical maximum work) may be greater than the total initial energy as determined by myothermic observation. In the straight-fibre muscles of frogs and tortoises the theoretical maximum work in shortening completely from the full resting length is, on the average, 1·22 times the initial heat, giving an over-all efficiency of $1\cdot22/2\cdot5 = 48\cdot8$ per cent. for the complete cycle of contraction and recovery (3, p. 259). It would appear therefore that in these comparisons of work and total energy we are coming back, in the case of man, to the same phenomenon as has long been of interest in the case of isolated muscle, namely, the very efficient transformation of chemical into mechanical potential energy in the "initial" process of contraction.

It is well known that in man no actual mechanical efficiency of more than about 25 per cent. can be attained: in such processes as bicycling and stair climbing there is an *optimum* speed, at and near which this efficiency is reached. The optimum speed is a compromise between the two factors (*a*) of "viscosity" which requires a slow movement to get much work and (*b*) of energy liberation during maintained contraction, which requires a rapid movement to save time and therefore energy. In discussing the "theoretical maximum work" one has eliminated the time element, only allowing enough time to develop the full force, and imagined a reversible cycle in which viscosity does not affect the result. In dealing with a tension-length curve in the isolated muscle we are doing what an engineer does with a *p-v* diagram, or a physicist with a Carnot cycle. We are dealing, not with an actual attainable efficiency, but with a theoretical maximum. This in fact we are doing also with a runner, for we cannot get a muscle actually to work without showing a "viscous" resistance to motion, and an efficiency of 38 per cent. against an external force is possible only if we could.

Thus, in this connection, we are dealing with muscular work in man as a thermodynamic problem. This needs emphasis, for it cannot be asserted that the working efficiency of human muscles, against a purely external resistance, is anywhere near the 38 per cent. found here; what can be asserted is that this theoretical treatment of the subject is as well justified, and as likely to prove fruitful, as that involving the "reversible process" and the Carnot cycle in physics.

The same high efficiency of transformation of chemical into mechanical energy in the initial process of muscular contraction, can be deduced in a general way from other well-known observations on man. In climbing a staircase an actual efficiency of 25 per cent. may be attained at a speed of 1 step in 1·2 second (4). At speeds far from this the efficiency is lower. Now there are two factors bringing down the efficiency: (*a*) the work done by a human arm muscle in 1·2 second is only about 75 per cent. of the theoretical maximum, owing to "viscosity," and (*b*) the energy required to maintain a contraction for 1·2 second is considerably more than that needed to keep it up for a short time only. It would seem almost certain that allowance for these two factors would bring the theoretical efficiency of stair climbing to a value at least as high as that deduced for running. The absence of violent extraneous movements, for example of the arms, would tend to make it more economical than sprint running, and we might expect an appreciably higher efficiency.

The Onset of Fatigue.—The very rapid onset of fatigue during sprint running, even in the fittest subjects, is well known, and from the above its explanation will be clear; the enormous amount of energy expended in running at top speed tells us that a runner may have gone half-way to complete exhaustion within 100 yards. This fatigue is manifested, not only by the magnitude of the oxygen debt, but by the slowing-up which occurs during a maximal effort continued for more than 7 or 8 seconds. This slowing-up is seen in the isolated muscle, and is a peripheral effect—it is not to be attributed to "fatigue" in the central nervous system.

Experiments have been made at Cornell on a variety of athletes, who have run 200 yards, at top speed throughout, being timed every 20 yards along the track. It is necessary to specify that the run is performed at top speed throughout, for the ideal way to run a 200-yards race is to maintain a constant speed, not to make a maximal effort the whole way. So much energy is wasted by developing an extra 0·5 yard per second in the early stages, that the speed falls, out of proportion, in the later ones. It can be shown mathematically that if the energy expended in running a given distance vary as the 2·8th power of the speed (Sargent (1), p. 17), then it is much more economical to run at a constant speed which is rather less than the greatest speed of an unfatigued subject. Athletic coaches realise this, and no 200- or 300-yards runner is advised to make a maximal effort throughout: skill is shown in the choice of speed which will land him at the finish in the minimum time.

For the present experiments, however, on fatigue to have any meaning a maximal effort must be made throughout, and a number of subjects have been good enough to volunteer their services. The results are given in the Tables below. The subjects were as follows:—

H.A.R.	A first-rate 100- and 220-yards runner.
C.M.W.	A quarter-miler capable of 49⅗ seconds.
O.	A half-mile runner.
C.	A half-mile runner, capable of 2 minutes.
S.............	A mile runner.
Ch.	A quarter and half-mile runner.
B.	A first-rate 1- and 2-mile runner.
P.	A good 2-mile runner.
H.	Aged 40, once a half-miler, similar to C.
F.	A poor runner, otherwise healthy and able-bodied.

The average speed of this group of men is given, at different distances, in the last row of Table III. We see that on the average the maximum speed is reached in 70 yards, that then it begins to fall, and by 190 yards has fallen as much as 12 per cent. If we may assume that the rate of energy expenditure of a fatigued muscle at a given speed is the same as that of a fresh muscle at that speed (a definite assumption for which no evidence is at present available), and that the energy liberated per second varies as the 3·8th power of the speed, then a fall of 12 per cent. in speed corresponds to a decrease of 39 per cent. in rate of liberating energy. Whether the assumption be justifiable or not, the decrease in speed, witnessed in every case, is very obvious.

In the average, widely different individuals have been grouped together. Some information of value comes from taking their cases separately. The most striking performance is that of H.A.R., who having done 100 yards in 9·88 seconds completed the second 100 yards in 9·57 seconds. He shows a 14 per cent. decrease in speed during the last 130 yards. On the other hand B., who is a first-rate long distance runner, and P., who is a good one, showed only a 4½ and a 6 per cent. decrease respectively. This is due, no doubt, in part to the fact that these two men are relatively immune to the effects of fatigue,

Table II.—Time in seconds for various distances in maximal effort.

Yards :	1	3	6	10	15	20	40	60	80	100	120	140	160	180	200
H.A.R.	0·38	0·75	1·205	1·715	2·275	2·760	4·595	6·380	8·125	9·885	11·695	13·550	15·455	17·425	19·455
C.M.W.	0·35	0·76	1·23	1·75	2·34	2·86	4·82	6·75	8·69	10·66	12·70	14·80	16·96	19·17	21·45
O.	0·42	0·83	1·32	1·85	2·46	3·03	5·07	7·10	9·28	11·27	13·39	15·58	17·87	20·19	22·57
C.	0·46	0·88	1·46	1·88	2·50	3·06	5·18	7·25	9·32	11·42	13·52	15·70	17·91	20·22	22·58
S.	0·40	0·86	1·37	1·91	2·56	3·15	5·32	7·45	9·56	11·62	13·87	16·26	18·47	20·90	23·19
Ch.	0·43	0·87	1·35	1·92	2·52	3·09	5·25	7·36	9·50	11·66	13·85	16·08	18·46	20·90	23·46
B.	0·38	0·79	1·29	1·84	2·49	3·09	5·29	7·52	9·72	12·10	14·23	16·51	18·84	21·16	23·56
P.	0·65	1·02	1·55	2·09	2·74	3·33	5·60	7·89	10·11	12·31	14·54	16·81	19·08	21·43	23·79
H.	0·54	1·10	1·64	2·24	2·90	3·54	5·84	8·10	10·39	12·70	15·05	17·47	19·97	22·55	25·27
F.	0·46	0·99	—	2·27	3·01	3·72	6·48	9·38	12·12	15·02	18·03	21·14	24·30	27·57	30·91

Table III.—Speed in yards per second at various distances.

(Note.—The speed at 30 yards is taken as the average speed over the interval 20 to 40 yards, etc.)

Yards :	30	50	70	90	110	130	150	170	190	Decrease, per cent. of maximum.
H.A.R.	10·90	11·14	11·46	11·37	11·04	10·78	10·50	10·16	9·85	14
C.M.W.	10·20	10·35	10·30	10·15	9·98	9·52	9·25	9·06	8·77	15
O.	9·8	9·85	9·2	9·6	9·5	9·1	8·7	8·6	8·8	10
C.	9·4	9·7	9·7	9·5	9·5	9·2	9·0	8·7	8·5	12
S.	9·2	9·4	9·5	9·7	8·9	8·5	8·9	8·6	8·4	13
Ch.	9·3	9·5	9·35	9·25	9·15	9·0	8·4	8·2	7·8	18
B.	9·1	9·0	9·1	8·4	9·0	8·8	8·6	8·6	8·7	4½
P.	8·8	8·9	8·9	9·05	8·95	8·8	8·8	8·6	8·5	6
H.	8·7	8·85	8·75	8·65	8·5	8·25	8·0	7·75	7·35	17
F.	7·25	6·8	7·3	6·8	6·6	6·4	6·3	6·1	6·0	18
Average	9·26	9·35	9·36	9·24	9·11	8·83	8·64	8·44	8·26	12

owing to their long-distance running; in part, also, it is probably due to the economical nature of their running, which fits them for long-distance work.

H.A.R., weighing about 165 pounds, propelling himself 11·46 yards a second, with a force equal to 0·815 of his body weight, is developing about 8·5 horsepower of mechanical energy. In running 100 yards at top speed he does about 40,000 foot-pounds of work, which with a 37·7 per cent. efficiency (see above) would leave him with an oxygen debt of 6·7 litres. One litre of oxygen used in recovery implies the removal of 6 grams of lactic acid. Thus 6·7 litres means 40 grams of lactic acid, a production of about 4 grams per second during running, nearly 1 gram per stride. It is this which causes the fatigue.

Summary.

1. The mechanical work done in sprint running against the viscous resistance of the muscles of the runner has been compared with the amount of oxygen used in recovering from the effort. The result shows a "mechanical efficiency" of about 38 per cent. as the average of 13 experiments on three subjects. The meaning of this high value is discussed.

2. In a man running 200 yards, at top speed throughout, fatigue begins to appear after 70 yards, and by the end the speed has fallen (on the average in 10 different runners) by 12 per cent. There are individual variations, trained long-distance runners showing a comparatively small diminution of speed in that distance.

3. This fatigue is due to the enormous rate of expenditure of energy in running at top speed. One subject who ran his first 100 yards in 9·88 seconds, and his second in 9·57 seconds, was developing 8½ horse-power at his maximum velocity (11·46 yards per second) and liberating more than 4 grams of lactic acid per second in his muscles.

Our sincere thanks are due to Prof. L. M. Dennis and the staff of the Baker Chemical Laboratory for their hospitality, and for the continual help we have received during the conduct of this work. Our most grateful thanks are due also to the athletic authorities at Cornell University, to Mr. R. Berry and Mr. J. F. Moakley, and particularly to the runners themselves whose friendly cooperation made the experiments possible.

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On the Occurrence of the Œstrous Cycle after X-ray Sterilisation. Part IV.—Irradiation of the Adult during Pregnancy and Lactation; and General Summary.

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(Communicated by Prof. J. P. Hill, F.R.S.—Received May 28, 1927.)

(From the Department of Physiology and Biochemistry, University College, London.)

CONTENTS.

PAGE

I. Introduction 51
II. Methods and Material 52
III. Synopsis of Experimental Animals 53
IV. Irradiation during Pregnancy 56
V. Irradiation during Lactation 57
    (*a*) Effect on lactation 57
    (*b*) Effect on recurrence of œstrus 59
VI. Summary 61
VII. General Summary 61

I. *Introduction.*

In the first two parts of this series (2, 3) it was shown that the complete destruction of Graafian follicles as organised units by irradiation of the young female does not inhibit the appearance of the œstrous cycle when puberty is subsequently reached, while in Part III (4) the cycle in the non-mated adult was shown to persist unchanged after sterilisation. The clear deduction from these results is that neither follicles nor corpora lutea are essential to the maintenance of the œstrous cycle in the unmated animal. Extensive investigation was first made on the sterilisation of young animals, because preliminary experiments had shown that irradiation of the adult, during the functional life of the persistent* corpus luteum at any rate, resulted in the permanent* histological survival of corpora lutea vera, and it was desired in the first place to investigate the œstrous cycle after sterilisation in the absence of such a complication. This anticipation was not altogether realised, because in certain cases the young follicles, sent into atresia by irradiation, produced corpora lutea atretica. In the majority of cases, however, the ovaries from animals sterilised at birth or at weaning time were free from tissue of follicular derivation.

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* The term "persistent" is applied to the corpora lutea of pregnancy and lactation which persist much longer than those of ovulation, while "permanent" is applied to the corpora lutea which appear to survive indefinitely after irradiation.

Since it seemed possible that the removal of follicular competition might cause the persistent corpus luteum to function for an abnormally long period, experiments on this question have been carried out, and the present paper deals with the irradiation of the adult during the functional life of the persistent corpus luteum (*i.e.*, irradiation during pregnancy and lactation), which results in the permanent histological survival of the corpora lutea vera, and with the effects of these structures on the recurrence of the œstrous cycle. The histology of the ovaries of the animals discussed in this paper is essentially similar to that found in the ovaries of non-parous adults after irradiation, and is considered in collaboration with Dr. Brambell elsewhere.

My thanks are again due to Prof. J. P. Hill, F.R.S., and Dr. F. H. A. Marshall, F.R.S., for advice and criticism. The mouse colony, from which the experimental animals were recruited, was maintained with the aid of a grant from the Government Grants Committee of the Royal Society, and afterwards with the aid of one from the Medical Research Council. Since this paper concludes the account of the œstrous cycle after X-ray sterilisation, I wish to take this opportunity of acknowledging my indebtedness to Prof. G. Elliot Smith, F.R.S., by whose permission the irradiations were carried out in the Department of Anatomy of University College, and to Dr. H. A. Harris for technical advice.

II. *Methods and Materials.*

The treatment of the animals, the dosage of X-rays employed, and the detection of œstrus, copulation and pregnancy have all been dealt with in detail in previous papers (1, 2).

Technique of Irradiation.—The order of dose required to produce sterility was originally ascertained by fixing all the variables except duration, and then gradually increasing the length of the exposures until a dose resulting in sterility was arrived at. The standards used for current wave-length, spark-gap, distance from anti-cathode, etc., were all given in Part I (2). Forty minutes' exposure of this standard intensity, which was found to produce sterility in a few cases, was found to give approximately ⅛ B tint with Levi's pastilles, while double this duration of exposure gave ¼ B tint. For some reason, however, even this increased dose failed to give consistent results on the lactating mouse, and two doses of this amount were given to the last batch of lactating animals irradiated (PC 28 to 42). ¼ B tint and ½ B tint were always given in two doses a week apart, and the date of irradiation is in all animals calculated from the first exposure.

Duration of Lactation Experiments.—In the experiments on the effect of the "permanent" corpora lutea on the efficiency and duration of lactation, the question arose as to how best to test the lactation. One litter can only be suckled for some 5 weeks, *i.e.*, for 2 weeks longer than is customarily allowed. In the end the arrangement hit upon was to allow the doe's own litter to be suckled for the usual 3 weeks and then to foster-mother another litter only a few days old. This second litter was weaned at the usual time, and another litter given to the doe. In this way the lactation could be prolonged for over 2 months. The usual end was for the third litter to show very poor growth, indicating cessation of lactation, and any further litter was generally eaten as soon as given to the doe. The various does suckled for different times, and odd litters were eaten when half grown, etc., but always the practice was to go on providing young until it was quite obvious that lactation had stopped. The precautions to be observed when foster-mothering have been described previously (1).

Identification of Animals.—The following index letters are used for the various series of animals described in this paper :—

S	Normal suckling does tested for possible duration of lactation in normal female.
SX.	Does used for testing duration of lactation after sterilisation.
PX.	Females irradiated during second half of pregnancy.
XVP.	Females irradiated soon after copulation.
PC.	Females irradiated during lactation.

III. *Synopsis of Experimental Animals.*

In the mouse persistent corpora lutea are found at two periods, *i.e.*, during lactation and pregnancy (1), and it was originally proposed to carry out the irradiations during pregnancy, and so to cause the corpora lutea of pregnancy to become histologically "permanent." Various difficulties arose, however, and the chief series of animals, upon which the effect of these permanent corpora lutea on the œstrous cycle was studied, were irradiated during lactation. In all, four series of animals are available for discussion in this paper. In the first place, five mice (SX series) were irradiated, both before and after parturition, the total irradiation being 30 minutes of the standard intensity. These mice were subsequently found to have been sterilised, the ovaries consisting 2½ months later of very little tissue except the "permanent" corpora lutea vera. These mice were used for investigating the effect of the absence of follicles and presence of these corpora lutea on the lactating capacity of the female, and are discussed in Section V (*a*).

Irradiation during Pregnancy.—In spite of the success in producing "permanent" corpora lutea in the SX series, the technique was obviously unsatisfactory on account of the irradiation being distributed between pregnancy and lactation, and the second series of mice were given the whole dose during the last week of pregnancy. The first four of these mice were given small doses for the purpose of ascertaining the effect on the young. Œstrous cycle histories are not available for any of these females, and their records may, therefore, be summarised in tabular form.

Table I.—Records of Animals Irradiated during Last Week of Pregnancy.

Animal.	Time of irradiation before parturition.	Length of irradiation.	Time of killing after irradiation.	Conditions of ovaries.
	Days.	Minutes.	Weeks.	
PX 1	10	10	20	Not sterile.
PX 2	5	10	19½	"
PX 3	1	10	13	"
PX 4	4	10	4	"
PX 5	3	40	6	Sterile.
PX 6	1	40	12	"
PX 7	Failed to give birth. Re-absorption (?)	40	4	Not sterile.
	Days.			
PX 8	4	40	—	—
PX 9	3	40	1	—
PX 10	1	40	3	Sterile.

Following these experiments 13 mice (the XVP series) were irradiated within a week of the discovery of the vaginal plug, and hence, since pregnancy follows copulation in about 80 per cent. of cases, during the first week of pregnancy in most instances.

The object of irradiation at this time was two-fold :—

(*a*) To sterilise the ovary during the early life of the persistent corpus luteum ; and

(*b*) To sterilise the embryos at an earlier age.

A brief review of the histories of these animals has been given in Part II, and they will be dealt with more fully in Section V of the present paper.

Irradiation during Lactation.—The experiments discussed above make it clear that irradiation during pregnancy presents certain difficulties. Chief among these is the fact that irradiation during the later stages of pregnancy catches the persistent corpora lutea during senility, while irradiation during the early part of pregnancy in most cases causes destruction of the embryos and the consequent failure of the corpora lutea to become really persistent. It seemed fairly clear, therefore, that irradiation during lactation, *i.e.*, during the functional life of the persistent corpora resulting from the immediate *post-partum* ovulation would have to be tried.

In all 42 mice were irradiated during lactation for the purpose of studying their subsequent œstrous history. Of these, the first 5 were given 40 minutes' exposure (*i.e.*, ⅛ B tint on Levi's pastilles), the next 22 80 minutes' exposure, while the last 15 had double this amount. Of the first 5 only 2 were found to have been sterilised, even though adequate time was allowed for this to take place. Of the following 22, 11 were found to be unsterilised, but 3 of these were dissected soon after irradiation, when sterilisation changes would not in any case have become complete. The last 15 of this series, which had the two 80-minute

Table II.—History of Animals Irradiated during Lactation but not Sterilised.

No. of animal.	Time of autopsy. Days after irradiation.	Dosage (*B tint*).	No. born before irradiation.	Time of autopsy after last œstrus. Days.	Notes.
PC 2	95	⅛	6	10	Had litter of three 11 days before autopsy.
PC 3	127	⅛	5	At parturition	Had litter of two when killed.
PC 4	78	⅛	7	0	Killed when vaginal plug found.
PC 11	114	¼	7	36	Had litter of two 37 days before autopsy.
PC 15	54	¼	5 suckled	16	Four normal cycles observed.
PC 16	122	¼	4 suckled	0	15 cycles observed.
PC 17	41	¼	4 suckled	6	—
PC 21	20	¼	6	—	Killed for histological material.
PC 22	27	¼	7	—	Killed for histological material.
PC 23	103	¼	6	0	13 normal cycles observed.
PC 24	103	¼	6	0	14 normal cycles observed.
PC 25	97	¼	6	0	14 normal cycles observed.
PC 26	87	¼	4	7	6 cycles observed.
PC 27	14	¼	4	—	Killed for histological material.
PC 29	68	½	9	17	4 cycles observed.
PC 32	68	½	9	5	5 cycles observed.
PC 38	56	½	9	5	3 cycles observed.

exposures, showed much more uniform results, only 3 of the animals having ovaries containing follicles when dissected. The first 5 animals were mated at the immediate *post-partum* œstrus, and four of them became pregnant, and were thus both pregnant and lactating when irradiated. All the series up to PC 14 were mated during the time that vaginal smear examinations were being made. The records of the unsterilised animals are given in tabular form above, and since it seems desirable not to include them in the discussion on the effects of removal of follicular competition on the history of the persistent corpus luteum, they will not be referred to again.

The sterilised animals of the series will be dealt with in Section V (*b*).

IV. *Irradiation during Pregnancy.*

As was mentioned in Part II, the most striking result of the irradiations during early pregnancy was that in almost all animals irradiation caused the pregnancy to terminate. The usual course of events was for œstrus to reappear soon after irradiation, and the following table gives the date of the appearance of the first œstrus after irradiation where the incidence is known.

Table III.—Return of Œstrus after Irradiation early in Pregnancy.

No. of animal.	Time of irradiation after conception.	First œstrus after irradiation.
	Days.	Days.
XVP 6	5	15
XVP 7	5	4
XVP 8	5	11
XVP 9	5	4
XVP 10	5	4

These results clearly indicate that the persistent corpus luteum of pregnancy is caused to undergo premature retrogression by exposure of the pregnant animal to X-rays. This, however, does not necessarily mean any direct effect of X-irradiation on the organ. The most probable hypothesis is that the fœtuses were adversely affected by irradiation, and that their death was followed in the ordinary way by the involution of the corpora lutea. Since in these animals the corpora lutea never become properly persistent, they throw no light on the effect of the removal of follicular competition on the persistent corpus luteum, and from this point of view the series of animals irradiated during early pregnancy must be considered rather a failure. Nevertheless, all the animals (except 11 to 13, which were killed at short intervals after irradiation) were subsequently examined for the occurrence of the œstrous cycle.

The making of vaginal smears was begun in XVP 1 to 5 four weeks after irradiation, at which time follicular degeneration should have become advanced if the irradiations had been effective. Of these 5 animals all except XVP 3 copulated, and XVP 1 and 2 were initially fertile, but later became sterile. Of the rest XVP 3 and 5 were found on subsequent histological examination to be sterile. XVP 3, however, was observed not to have a regular cycle, one isolated œstrous period only being observed. XVP 4 had a series of normal œstrous periods accompanied by copulation, but was not sterilised. Vaginal smearing was begun on XVP 6 to 10 before the irradiations and was continued for considerable periods afterwards. Of these animals XVP 10 died before its œstrous history could be satisfactorily determined, and XVP 6 and 8 copulated and became pregnant sufficiently long after irradiation to preclude the possibility of sterilisation having been effected. XVP 7 and 9, though conceiving at the immediate post-irradiation œstrus (see Table III), were subsequently found histologically to be sterile.

V. *Irradiation during Lactation.*

(*a*) *Effect on Lactation.*—The statement has often been made that in the cow double ovariotomy during lactation will prolong considerably, if not indefinitely, the period during which lactation will last, and the experiments to be described in this section were arranged in order to find out whether any comparable result could be obtained by X-ray sterilisation. It was originally hoped actually to sterilise a cow during lactation for this purpose, and by the courtesy of Captain J. Golding and Dr. Williams, of the National Institute for Research in Dairying, Reading, this was attempted. The relative weakness of the portable X-ray set, however, proved a severe handicap, and this taken in conjunction with the movement of the animal, the position of the ovaries, and the thickness of the flesh on the lumbar region, caused the project to be abandoned after two exposures.

Control Animals.—When the same type of experiment was tried on mice, it first of all became necessary to find some means of testing the utmost duration of lactation in such an animal, where no direct assessment is possible, and as explained in Section II, repeated foster-mothering of young litters was resorted to. It was also necessary to investigate how long the normal mouse would lactate if necessity and opportunity arose. This control experiment had a rather surprising result, it being found that a female mouse can rear at least two foster-litters following the suckling of her own litter. The individual records of the seven normal suckling females used in this experiment are given in the following table :—

Table IV.—Prolonged Suckling in Normal Mice.

Mouse.	No. of litters reared.	Time suckling.	Fate of last litter.
		Days.	
S 1	3	61	Fourth litter died in 2 days.
S 2	3	63	Fourth litter died in 1 day.
S 3	2	34	Three litters given in last week, but all eaten by doe.
S 4	2	40	Third litter was suckled for a few days and then died of malnutrition.
S 5	1	27	Second litter suckled for a few days then all died of diarrhœa.
S 6	2	52	Third litter was suckled 8 days and then eaten.
S 7	4	89	Fifth litter died in 3 days.

The foster young were not all given at birth ; young a few days' old stand the change better.

S 3 ate three sets of young in one week, and it would appear that the animal could have reared further young, but would not. The 34 days of lactation in this case, therefore, cannot be considered as representing the utmost duration of lactation. S 5, which died of diarrhœa after 27 days, has also to be excluded from the series, so that the remaining 5 cases give total periods of lactation ranging from 40 days to 89 days, or roughly from 6 weeks to 13 weeks. The condition of the doe is presumably an important factor in this variation. The point that this control experiment does show, however, is that the normal mouse can suckle for much longer than it is habitually called upon to do ; consequently, experiments on the duration of lactation would need to show very striking results before much weight could be attached to them.

Irradiated Animals.—The histories of the experimental animals sterilised during the early part of lactation are given below (see Table V).

Excluding SX 3, which died prematurely, the periods of lactation in these sterilised animals varied from 56 days to 86 days, three of the periods, however, being above 80 days. The actual average length of time during which the female will lactate is greater in the sterilised animals than in the normal, but in view of the variation in the individual performances of both sets, it seems hard to attach much significance to this empirical result.

Table V.—Prolonged Suckling in Sterilised Females.

Number of animal.	Irradiation during pregnancy.	Irradiation during lactation.	Number of litters reared.	Duration of suckling.	Fate of last litter.
				Days.	
SX 1	10 mins. 3 days and 10 mins. 1 day before parturition	10 mins. 3 days after parturition	4	82	Fifth litter died in 3 days.
SX 2	10 mins. 3 days and 10 mins. 1 day before parturition	10 mins. 3 days after parturition	2	56	Third litter suckled for 11 days, when doe died.
SX 3	10 mins. 9 days, 10 mins. 5 days, 10 mins. 2 days before parturition	—	1	31	Doe died when suckling second litter.
SX 4	10 mins. 2 days before parturition	10 mins. 2 days, 10 mins. 5 days after parturition	4	88	Fifth litter died in 3 days.
SX 5	10 mins. 2 days before parturition	10 mins. 1 day and 10 mins. 4 days after parturition	4	86	Fifth litter died in 2 days.

(*b*) *Effect on Recurrence of Œstrus.*—After the immediate *post-partum* period no further signs of œstrus are normally observed in the lactating mouse for some 3 to 4 weeks. The actual length of the long lactation diœstrus was found (Parkes, 1) to average about 25 days, and this inhibitory effect has been shown (Parkes and Bellerby, 5) to be regulated by the ovary, and almost certainly by the corpora lutea of lactation, but the mechanism whereby lactation produces this effect is unknown. Prolonged lactation is not accompanied by prolonged œstrus-inhibition. The length of the lactation diœstrus was found to be virtually the same whether suckling ended at the normal time (3 weeks) or whether it was prolonged (1). This indicated a maximum functional life of the persistent corpus luteum of lactation, but the factors causing the decline of activity were again unknown. Since follicular competition seemed to be a possible cause, it was clear that the destruction of the follicles by X-rays during lactation could be used to test this point.

In view of the long histological persistence of corpora lutea vera in animals sterilised during lactation, it was originally thought that they might still retain functional activity for a very long period, and the first animals of the PC series were therefore not examined immediately after lactation. The œstrous histories of the animals known to have been sterilised are summarised in the following table:—

Table VI.

No. of animal.	No. born.	Time of irradiation after parturition.	Dosage (B tint).	Observations begun (time after parturition).	Time observed.	No. of œstrous periods.
		Days.		Days.	Days.	
PC 1	7	1	⅓	53	10	1 (copulation).
PC 5	3	1	⅓	53	40	3 (copulation).
PC 6	8	4	¼	67	58	3 (copulation).
PC 7	6	2	¼	65	58	1 (copulation).
PC 8	7	4	¼	67	58	2 (copulation).
PC 9	5	2	¼	65	58	3 (copulation).
PC 10	3	1	¼	64	58	1 (copulation).
PC 12	5	7	¼	64	58	1 (copulation).
PC 13	3	5	¼	62	58	1 (copulation).
PC 14	6	4	¼	61	58	1 (copulation).
PC 18	—	—	¼	—	24	3 (unmated).
PC 19	—	1	¼	32	72	7 (unmated).
PC 20	—	1	¼	32	13	1 (unmated).

These results show that the œstrus-inhibiting function of the persistent corpora lutea of the sterilised ovary does not extend indefinitely. The animals whose histories are summarised in the following table were therefore examined regularly from before the end of lactation up to 6–7 weeks after irradiation. In this table (Table VII) the time of appearance of the post-lactation œstrus is given in the fourth column, while the number of œstrous periods observed, including this one, is given in the next column. The cycles referred to exclude the one comprising the long lactation diœstrus and the following œstrus.

These results show that, though there may be a slightly longer than normal œstrus-inhibiting activity on the part of the corpora lutea of lactation, when they are freed from follicular competition and are rendered histologically "permanent," the effect is not very obvious, and may in fact lack real significance. This indicates that the functional life of even the persistent corpus luteum of the mouse is strictly limited, and even under what might be considered *optimum* conditions, the normal length of activity is not materially prolonged.

Table VII.

No. of animal.	No. born.	Time of irradiation. Days after parturition.	Time of re-appearance of œstrus. Days after parturition.	No. of œstrous periods observed.	Length of cycles.	Average length.	Time of killing. Days after irradiation.
						Days.	
PC 28	8	5	36	3	1-17-11	14·0	68
PC 30	8	6	30	6	5-8-10-10-6	7·8	68
PC 31	8	5	28	4	6-10-10	8·6	68
PC 33	8	5	30	2	8	8·0	68
PC 34	5	0	23	5	8-7-10-8	8·2	68
PC 35	11	9	30	5	5-11-7-8	7·8	56
PC 36	7	10	29	3	4-11	7·5	42
PC 37	8	2	30	2	9	9·0	48
PC 39	10	2	31	3	13-13	13	58
PC 40	9	3	42	1	—	—	56
PC 41	8	3	38	2	5	5	56
PC 42	8	1	43	1	—	—	56
Average....	8·2	—	32·5	—	—	—	—

VI. *Summary.*

I. Irradiation during early pregnancy usually results in reabsorption or abortion, so that the corpora lutea present at this time fail to become true persistent corpora lutea of pregnancy, and œstrus supervenes soon after the irradiation.

II. Irradiation during lactation though causing follicular disintegration does not affect the persistent corpora lutea of lactation, and these are caused to become histologically " permanent."

III. These corpora lutea, though " permanent," cannot be shown to retain their œstrus-inhibiting power for any appreciable period longer than the functional life of the normal persistent corpus luteum.

IV. The possible duration of lactation is not affected by sterilisation at the beginning of the lactation period.

VII. *General Summary.*

The series of paper which is concluded above has dealt with the sterilisation of the ovary of the mouse by exposure to X-rays, and with the fact that the obliteration of the cyclic structures of the ovary does not affect the cyclic occurrence of œstrous symptoms in the accessory organs. Irradiation of the young female at three weeks old (Part I) results in the complete degeneration of ova, granulosa cells and theca interna, where it has differentiated; and the ovary subsequently comes to consist almost entirely of extra-follicular tissue, *i.e.*, proliferations of the germinal epithelium. In animals irradiated at birth (Part II) the usual course of events is similar to that found in those irradiated at three weeks old, but in some few instances the proliferation from the germinal epithelium becomes extremely luteal-like. When the animals irradiated before puberty become adult, the normal cyclic œstrous changes are found in the accessory organs in all cases except those few in which the ovary has undergone luteal degeneration.

The fact that the proliferations from the germinal epithelium appear to elaborate the hormone required for the production of œstrus suggested that irradiation of the adult might cause a temporary cessation of the cycle between the time of irradiation and the time of development of the proliferations from the epithelium. The study, however, of a large number of irradiated adults (Part III) showed that irradiation of the adult, though obliterating the follicles as such, does not eliminate follicular tissue from the ovaries; in correlation with this it was found that no post-irradiation proliferation from the germinal epithelium took place. The œstrous cycle in the irradiated adult is perfectly normal as regards the extra-ovarian features, and no hiatus can be detected either immediately after irradiation, or later when follicular disintegration had become complete.

These results make it perfectly clear that the alternating maturation of Graafian follicles and corpora lutea is not essential for the production of the cyclic œstrous changes in the accessory organs, and that the periodicity of œstrus is not therefore determined by the periodic follicular maturation. Two points then are raised by these experiments: (*a*) what is the mechanism whereby the periodicity of the œstrus stimulus is brought about? and (*b*) by what means are œstrus and ovulation synchronised? The first of these problems still remains to be studied, but it has been tentatively suggested that the periodicity of œstrus results from the periodic attainment of a threshold value on the part of the œstrus-producing hormone. The second problem has been partially solved, and it seems highly probable that the synchronisation of ovulation and œstrus is brought about by their being dependent on a common stimulus.

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Transmission Experiments with Chinese Kala Azar.

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(Members Kala Azar Commission of the Royal Society.)

(Communicated by Dr. H. H. Dale, Sec. R.S.—Received June 14, 1927.)

In the course of our experiments on the development of flagellates in sandflies fed on animals infected with Chinese Kala Azar (Patton & Hindle, 1927), numerous attempts were made to infect hamsters by means of these insects. The life-history of the flagellate in *Phlebotomus major*, in which insect there is a rapid growth of the parasites towards the anterior part of the alimentary canal, ending with infection of the mouth parts, strongly suggests that the parasite is transmitted by the bite of an infected fly, consequently many of our experiments consisted in feeding sandflies on infected animals and subsequently on normal ones. The possibility of other methods of infection was kept in mind, however, and on the analogy of *Trypanosoma lewisi* in the rat flea, attempts were made to infect animals by feeding them with sandflies containing flagellates, as it is conceivable that the infection may reach the body through the alimentary canal. In addition infection might be produced by parasites, set free by crushing the insect, entering the body either by means of the open wound caused by the bite of the fly, or through an excoriated part of the skin.

The results of preliminary experiments with culture flagellates (Hindle & Patton, 1926) showed that, although the hamster could be readily infected by intraperitoneal inoculation of these forms, the subcutaneous and percutaneous methods of infection succeeded only occasionally. From these experiments one would not expect the bite of an infected sandfly to produce infection except in rare cases, for obviously the parasites are only introduced subcutaneously when the insect feeds, and therefore, in most instances, the contents of infected flies were inoculated intraperitoneally into hamsters in order to see whether the flagellates were infective or not. Most of our experiments in 1926 were made with *Phlebotomus sergenti*, which is not such a favourable host as *P. major* var. *chinensis*, and in view of the fact that flagellates were never observed in the pharynx of the former species, it is not surprising that all feeding experiments gave negative results. *P. major* has a comparatively short season, and the few experiments we were able to perform are not sufficient to indicate the efficiency of this species as a carrier.

Methods.

In all cases the sandflies were fed in the manner described previously (Patton and Hindle, 1927) and the numbers of the experiments refer to the tables contained in that article. As a rule the sandflies were dissected only after they had died, and therefore in some cases the virulence of the contained flagellates may have been diminished as a result of the death of the host. When a fly was dissected and found to contain active flagellates the gut contents, suspended in saline, were collected in a glass capillary tube and inoculated into the peritoneal cavity of a normal hamster. Usually the contents of several flies were inoculated into the same animal in order to increase the chance of infection.

After the flies had been fed on an infected animal any subsequent feeds were made on normal hamsters, but very few of the flies re-fed more than twice. Hamsters which had been exposed to infection in any way were kept for about six months in order to allow ample time for the disease to develop. When killed, films were made from the liver, spleen and bone marrow of each hamster, and in addition cultures on blood agar were made from the liver and spleen. In order to reduce the chance of overlooking any scanty infections, all the films were examined in turn by three members of the staff, so it is unlikely that any infections were not detected. Moreover an infected animal generally shows a considerable enlargement of the spleen and the parasites are present in such numbers that it is almost impossible to miss seeing them.

Experiments with Phlebotomus major *var.* chinensis.

(*a*) *Feeding Experiments.*—Only four hamsters were used in these experiments. These were exposed to the bites of sandflies which had fed on infected hamsters respectively four, five, six and seven days previously. In every case the flies were found to be heavily infected with flagellates when subsequently examined. The hamsters all remained negative after intervals of approximately six months.

(*b*) *Experiments in which the Insect Flagellates were rubbed into the Excoriated Surface of the Skin.*—Thirteen hamsters were used and in every case part of the skin was shaved and also pricked with a fine needle. Subsequently infected sandflies were crushed on to the surface and rubbed into the skin. The flies used in these experiments had been fed on an infected animal respectively five, six and seven days previously, and in all except two cases were proved to contain flagellates. All the hamsters remained negative after incubation periods up to six months.

(*c*) *Sandflies Administered by Mouth.*—In these experiments infected sandflies were swallowed by hamsters. In six cases the flies were crushed inside the mouth of the hamsters before being swallowed, and in these insects flagellates were actually observed by microscopic examination of part of the crushed contents. In the remaining four animals the flies were swallowed whole and consequently could not be examined for flagellates, but there is strong presumptive evidence that they must have been infected. The sandflies had fed respectively six and seven days previously on an animal infected with *Leishmania*. All the hamsters remained negative.

(*d*) *Intraperitoneal Inoculation of Flagellates from Gut of Sandfly.*—A total of 16 hamsters were inoculated intraperitoneally with the gut contents of one or more sandflies fed on an infected animal, respectively two, three, four, five, six, eight and ten days previously. In every case flagellates were present in the midgut of the insects, and after five days' interval the parasites, as a rule, could also be seen in the pharynx. All the hamsters remained negative after varying incubation periods ranging up to six months.

Experiments with Phlebotomus sergenti.

(*a*) *Feeding Experiments.*—Twenty-nine hamsters were subjected to the bites of sandflies that had been fed only once previously. In every case the insects had been fed on a heavily infected animal and the interval between the first and second feeds varied from two to eight days. All the hamsters remained negative.

Sixteen hamsters were subjected to the bites of sandflies that had been fed twice previously, once on an infected animal and subsequently on normal individuals. The interval between the infected feed and the third one varied from four to six days, as it is possible to feed *P. sergenti* every two or three days. All the hamsters used in these experiments remained uninfected.

Four hamsters were exposed to the bites of sandflies that had fed three times previously, the first meal on an infected animal and the subsequent meals, with one exception, on normal individuals. The interval between the infected meal and the fourth feed was either seven or eight days. All the hamsters remained negative.

One hamster was exposed to the bite of a sandfly that had fed four times previously. The first and second feeds were on infected hamsters; the succeeding three feeds on normal animals. The interval between the first and fifth feed was nine days. The hamster bitten by the above-mentioned sandfly, the only one in the laboratory which fed five times, remained negative after an incubation period of 27 weeks.

In all except two of the above described experiments one or more of the sandflies were found to contain flagellates in the midgut, but in no case were parasites observed in the pharynx of any insect dissected.

(*b*) *Sandflies Administered by Mouth.*—Nine hamsters swallowed varying numbers of sandflies three to six days after the insects had been fed on an infected animal. In some cases the flies were crushed in the mouth and it was possible to verify the presence of flagellates in the material that was swallowed. In all the other cases it is practically certain that each hamster had swallowed infected sandflies, judging from the dissection of other insects belonging to the same batches as those fed to the hamsters. All these animals remained negative after incubation periods up to six months.

(*c*) *Intraperitoneal Inoculation of Flagellates from Gut of Sandfly.*—In all, 77 hamsters were inoculated intraperitoneally with saline suspensions of the gut contents of one or more sandflies containing flagellates. 46 were inoculated with the contents of flies that had fed only once—on an infected animal—and then been kept for a period ranging from two to seven days. Four of these hamsters became infected as a result of the inoculation, and the particulars of these experiments are given below.

Twenty-seven hamsters were inoculated with the contents of sandflies that had fed twice, once on an infected animal and subsequently on a normal one, and then been kept for periods of four to ten days after the infective meal, whilst four hamsters were inoculated with flies that had fed three or more times and been kept for seven to ten days after the infective meal. None of these 31 inoculated hamsters showed any signs of infection with *Leishmania* although kept for sufficiently long incubation periods.

The particulars of the four positive experiments—the only ones we obtained with sandflies duing the season—are as follows :—

Experiment S 71.

First feed :—

21.8.26.—Fed 60 *P. sergenti* on hamster No. 212 and kept the flies at a temperature of 20° C.

23.8.26.—Dissected four of these flies. Three contained flagellates in the midgut and one was negative. The contents of these flies were inoculated intraperitoneally into hamster No. 571, which was killed on 18.2.27. On examination the spleen was found to be much enlarged and the liver, spleen and bone marrow all contained large numbers of parasites.

24.8.26.—Dissected two flies. Both heavily infected with flagellates in midgut. Inoculated the contents of these flies into hamster No. 576, which was killed on 18.2.27, and showed no signs of infection.

25.8.26.—Dissected four flies. Three were heavily infected in the midgut and one negative. The contents were inoculated into hamster No. 578, which was killed on 18.2.27 and found to be heavily infected with *Leishmania.*

On the same date 22 of these flies were swallowed by hamsters Nos. 579 and 580, each receiving 11. Both these hamsters were still negative on 18.2.27, when killed and examined.

Second feed :—

24.8.26.—Twelve flies were re-fed on normal hamster No. 577, which was still negative on 18.2.27 when killed and examined.

26.8.26.—Two of these flies dissected. Both heavily infected in the midgut. Inoculated the contents into hamster No. 592, which was negative on 19.2.27 when killed and examined.

27.8.26.—Dissected four flies. Two heavily infected in midgut and two negative. Inoculated contents into hamster No. 599, which was negative on 19.2.27 when killed and examined.

28.8.26.—One dissected. Heavily infected in midgut.

Third feed :—

26.8.26.—Five flies re-fed on a normal hamster No. 596, which was negative on 19.2.27 when killed and examined.

28.8.26.—One dissected. Heavily infected in midgut.

29 & 30.8.26.—Flies refused to feed. One escaped.

31.8.26.—Remaining three flies dissected. Two were negative and one positive in the midgut. The contents were inoculated into normal hamster No. 605, which was still negative when killed and examined on 21.2.27.

Experiment S 74.

First feed :—

24.8.26.—Fifty-three *P. sergenti* fed on infected giant hamster No. 252 and kept at 30° C.

26.8.26.—Seven flies dissected, of which six were positive in the midgut and one negative. The contents were inoculated into a normal hamster No. 595, which was killed on 19.2.27. On examination the spleen was found to be enlarged and Leishman-Donovan bodies were present in the liver, spleen and bone marrow.

27.8.26.—Five flies dissected. One positive in midgut and other four negative. Inoculated contents into normal hamster No. 598, which was negative when examined on 19.2.27.

28.8.26.—Two dissected. Both negative.

29.8.26.—Five dissected. All negative.

Second feed :—

27.8.26.—Re-fed 34 flies on normal hamster No. 601, which was negative when killed and examined on 21.2.27.

28.8.26.—Five dissected. One positive in midgut and four negative.

30.8.26.—Four dissected. One positive and three negative.

Third feed :—

29.8.26.—Re-fed 18 flies on normal hamster No. 606, which was still negative when killed on 21.2.27.

31.8.26.—Five dissected. All negative.

1.9.26.—Six dissected. All negative.

Fourth feed :—

31.8.26.—Three flies re-fed on normal hamster No. 611, which was negative when examined on 22.2.27.

2.9.26.—Three dissected. All negative.

In the above experiment the infection seems to have died out in the sandflies after the second feed.

Experiment S 75.

First feed :—

26.8.26.—Fed 75 *P. sergenti* on infected hamster No. 212 and kept at 30° C.

28.8.26.—Six flies dissected. Five negative, but flies degenerated, and one positive.

29.8.26.—Eight flies dissected. Six contained flagellates in midgut, the other two were negative. The contents were inoculated into normal hamster No. 604, which died on 26.1.27 heavily infected with *Leishmania.*

30.8.26.—Twelve flies dissected. Seven negative, and five very heavily infected in midgut. The contents were inoculated into normal hamster No. 608, which was still negative on 21.2.27 when killed for examination.

Second feed :—

29.8.26.—Re-fed 35 sandflies on normal hamster No. 607, which was negative on 21.2.27 when killed for examination.

30.8.26.—Six flies dissected. Five negative and one positive in midgut.

31.8.26.—Three flies dissected. Two negative and one positive, but only one flagellate seen.

1.9.26.—Four flies dissected. One negative and three very heavily infected in midgut. The contents were inoculated into normal hamster No. 613, which died on 25.9.26 without showing any signs of infection.

Third feed :—

31.8.26.—Re-fed 14 sandflies on normal hamster No. 612, which was negative when killed on 22.2.27.

2.9.26.—Four flies dissected. Three negative and one heavily infected in midgut. The contents were inoculated into normal hamster No. 611, which was negative when killed on 22.2.27.

3.9.26.—Five flies dissected. All negative, but flies somewhat degenerated having died overnight.

4.9.26.—Three flies dissected. All negative.

Fourth feed :—

3.9.26.—Re-fed two sandflies on normal hamster No. 616, which was negative when killed on 22.2.27.

4.9.26.—One fly dissected. Very heavily infected in the midgut. The contents were inoculated into normal hamster No. 618 which was negative when killed on 22.2.27.

5.9.26.—Remaining fly dissected. Negative.

The above three experiments have been given in detail, as they are typical examples. Since all other transmission experiments gave negative results it seems unnecessary to give full particulars of them, as they resemble the above except in minor details.

Discussion of Results.

The negative results of our feeding experiments with *Phlebotomus major* and *P. sergenti* agree with those obtained in India by Shortt, Barraud and Craighead (1927, A), who fed a total of 152 infected *P. argentipes* on sixty experimental animals without producing any infection. It should be remembered, however, that comparatively few parasites can be inoculated when a sandfly bites, and judging from the results of our experiments with culture flagellates (Hindle and Patton, 1926), the subcutaneous method of inoculation is only about one-tenth as effective as the intraperitoneal.

If we consider the results obtained by the intraperitoneal inoculation of flagellates from the alimentary canal of infected sandflies, again there is a resemblance between those obtained in India and the above-described experiments with *P. sergenti* in China. Shortt, Barraud and Craighead (1927, B) infected one white mouse out of a total of 53, by the intraperitoneal inoculation of the contents of three *P. argentipes*, two, nine days after the infective feed, and one, eleven days. As we were successful in four cases out of a total of 46, the hamster would seem to be more susceptible than the white mouse, and the results of other inoculation experiments support this view.

Considering the small proportion of animals that have been infected by the intraperitoneal inoculation of the whole of the gut contents of several sandflies containing flagellates, it is not surprising that up to the present feeding experiments should have given negative results. Our inoculation experiments with *P. sergenti* resulted in an average of only one in 11·5 hamsters becoming infected, and in feeding experiments this proportion should be divided by at least 10, as the sandfly obviously can only introduce the parasite subcutaneously, giving a ratio of only 1 in 115. At first sight this might seem evidence against the view that the parasite is transmitted by the bites of sandflies, but it should be remembered that when present these insects often occur in very large numbers, and a person may easily be bitten by a hundred or more in the course of a single night.

Reference should be made to one factor which seems to be of importance in transmission experiments, namely, the type of infection in the vertebrate host. In our previous account of the development of *Leishmania* in Chinese sandflies (Patton and Hindle, 1927), we have referred to the results obtained by feeding sandflies on human cases of the disease. Four out of twelve flies fed on one patient showed development of flagellates ; one out of fifty-three flies fed on a second patient ; and numerous flies fed on five other patients, each with well-marked symptoms of the disease of long duration, all remained negative.

As regards the infectivity of the flagellate infection there are indications of similar individual variations, which must have been derived from the vertebrate host. In the series of experiments which have been described above, eight infected hamsters were used on which to feed the sandflies, and with the exception of Giant Hamster No. 252, which was inoculated with culture flagellates, all the hamsters were infected with the same strain of *Leishmania*, a Peking strain kindly supplied by Drs. Young and Hertig. In every case parasites could be seen in the peripheral blood. In three out of the four positive experiments on the infectivity of the gut flagellates, the sandflies had been fed on the same hamster, No. 212, and in the fourth case the flies had been fed on Giant Hamster No. 252.

Summarizing the results obtained by the intraperitoneal inoculation into hamsters of the contents of flies that had fed on different animals without being re-fed, it seems that in some way the virulence of the parasite has been enhanced in No. 212, and to a lesser degree in No. 252. Details of the hamsters used in these experiments are shown in the following records :—

Hamster No. 80. 9.3.26.—Inoculated intraperitoneally with Peking strain. Fed sandflies on dates ranging from 28.5.26 to 12.8.26. Fourteen hamsters were inoculated on different occasions, four with the gut contents of infected *P. major*, and ten with the contents of infected *P. sergenti*. All the hamsters remained negative.

Hamster No. 81. 9.3.26.—Inoculated intraperitoneally with Peking strain. Fed sandflies on dates ranging from 28.5.26 to 9.8.26. Thirteen hamsters were inoculated on different occasions, four with the gut contents of infected *P. major*, and nine with the contents of infected *P. sergenti*. All the hamsters remained negative.

Hamster No. 99. 9.3.26.—Inoculated intraperitoneally with Peking strain. Fed sandflies on dates ranging from 25.6.26 to 14.8.26. Ten hamsters were inoculated on different occasions, two with the gut contents of infected *P. major* and eight with the contents of infected *P. sergenti*. All the hamsters remained negative.

Hamster No. 116. 23.12.25.—Inoculated intraperitoneally with Peking strain. Fed sandflies on dates ranging from 14.8.26 to 19.8.26. Four hamsters were inoculated on different occasions, two with the gut contents of infected *P. major* and two with the contents of infected *P. sergenti*. All the hamsters remained negative.

Hamster No. 119. 23.12.25.—Inoculated intraperitoneally with Peking strain. Fed sandflies on 15.8.26 and 16.8.26. Three hamsters were inoculated on different occasions with the gut contents of infected *P. major*. The three hamsters remained negative.

Hamster No. 212. 12.3.26.—Inoculated intraperitoneally with Peking strain. Fed sandflies on dates ranging from 16.8.26 to 26.8.26. Eight hamsters were inoculated on different occasions, one with the gut contents of infected *P. major*, and seven with the contents of infected *P. sergenti*. Three of the latter hamsters became infected with *Leishmania*, whilst five remained negative.

Hamster No. 221. 12.3.26.—Inoculated intraperitoneally with Peking strain. Fed sandflies on 16.8.26 and 18.8.26. Four hamsters were inoculated on different occasions with the gut contents of infected *P. sergenti*. All the hamsters remained negative.

Giant Hamster No. 252. 6.4.26.—Inoculated intraperitoneally with Peking strain. Fed sandflies on dates ranging from 18.8.26 to 24.8.26. Six hamsters were inoculated on different occasions with the gut contents of infected *P. sergenti*. Five hamsters remained negative and one became infected with *Leishmania*.

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Experiments concerning the Question of Secretion of Phenolsulphonephthalein by the Renal Tubule.

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(Communicated by Dr. H. H. Dale, Sec. R.S.—Received June 15, 1927.)

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[Plate 1.]

Among the most important methods of study which have been applied to the question of secretion by the renal tubule are those which involve the use of dyes. Heidenhain revived the Bowman theory of secretion, largely because of his observations relating to the elimination of indigo carmine—observations which form the beginning of a voluminous and confusing literature. The importance of complete information concerning the mode of excretion of certain dyes can scarcely be denied: they resemble most of the normal urinary constituents in that the concentration in which, after injection, they appear in the urine is higher than that in the blood. As has frequently been argued, if the mechanisms which take part in the excretion of these substances can be identified, light will be thrown upon the processes of normal urine formation. The experimental advantage which derives from their colour is obvious.

Phenolsulphonephthalein (phenol red) was introduced as a clinical test of renal function by Rowntree and Geraghty(1) in 1912. Among their experiments was one which convinced them that the tubule was actively concerned in its elimination. After section of the renal arteries in the frog, a phenol-red solution was perfused through the renal portal vessels at a pressure of 35 cm. water. The resulting appearance of the dye in the ureteral fluid convinced them that it had been "secreted" by the tubule. It was soon shown by Bainbridge, Collins and Menzies(2), however, that such high pressure in the renal portal vessels may drive fluid through the efferent vessels into the glomeruli; thus their "proof" that the glomeruli had not been involved in the observed excretion was invalidated.

Ten years later, direct observations of the glomeruli in the living frog's kidney, together with inspection of fluid collected during life from capsules

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of Bowman, proved beyond doubt that phenol red is separated from the blood as it passes through the glomerulus([3, 4]). At the same time, also, direct proof of selective re-absorption in the renal tubule was supplied. Hence for a time it appeared that the elimination of phenol red in the urine could be explained without assuming active secretion of the dye by the tubule.

More recently, belief in a secretory factor in this connection has been revived. Marshall and Vickers([5]) found (1) that, when injected into the circulation of a dog which is secreting no urine because of low blood pressure, phenol red accumulates in the cortex of the kidney in far higher concentration than that in the blood or in any other tissue; (2) that only about 40 per cent. of phenol red added to dog's plasma is free, *i.e.*, filterable through collodion; and (3) that under certain conditions after injection the substance in its free form is not present in sufficient concentration in the arterial blood to account for the quantity eliminated in the urine, even if glomerular filtration of all the plasma is assumed to occur.

The conclusions drawn from these results have been widely accepted. Starling and Verney([6]) found that the perfused dog's kidney eliminated urine poorer in chlorides, richer in urea and phenol red, than the blood plasma which flowed through its vessels. Poisoning of the kidney by cyanide caused urinary chloride to increase; urea and phenol red to decrease. They believed that cyanide inhibited the re-absorptive power of the tubule for chlorides, and the secretory power for urea and phenol red.

Issue was taken with Marshall's conclusions by Bieter and Hirschfelder([3]). Using the method of direct observation of the frog's kidney during life, which was introduced by Richards and Schmidt, they watched the accumulation of phenol red in the tubules following subcutaneous injections. In areas of the kidney which had been deprived of glomerular circulation, they were unable to see accumulation of colour in the tubules, despite the fact that the tubules in these areas still received blood (and phenol red) by way of the renal portal system. Marshall([7]) replied that Bieter and Hirschfelder observed only the ventral surface of the kidney; that the proximal convolutions of the tubule are the parts concerned in the secretion of phenol red, and these are situated in the dorsal region. Most recently, Bieter and Hirschfelder([8]) have published a preliminary account of experiments by a somewhat more intricate method, which gave further results not in accord with Marshall's view.

This review shows that while the study of phenol red has yielded influential support of the doctrine of secretion by the renal tubule, the question is by no means settled, even as it relates to this one substance. We know from direct experiment that fluid containing chloride, sugar, urea, and foreign substances, including dyes, if these are present in the blood, is separated from the blood in large amount as it passes through the glomerulus; we know from direct experiment that re-absorption occurs in the tubule; but we do not know with comparable certainty that any substance is actively secreted into the tubule by the epithelium which forms its wall. Efforts are described in this paper to arrive at an unequivocal experiment upon this question. Phenol red was chosen as the subject of study because of the discussion which is current about it, and because of the ease with which it can be identified and estimated.

I.

In so far as we are aware, it has not yet been shown by direct conclusive experiment that phenol red can, under any circumstances, pass through the wall of the tubule of the living kidney into its lumen, either by diffusion or secretion. The necessity of testing this question is obvious. The experiments described in this section represent the chief steps in a search for methods which might supply such a demonstration. Our first experiments were made on rabbits: we soon turned to the frog's kidney because the minute details of its structure are accessible to microscopic observation during life, and because of its equipment with a renal portal vascular system.

1. *Application of Phenol Red to the Surface of the Rabbit's Kidney after Decapsulation.*—When the rabbit's kidney is carefully decapsulated, the surface which is exposed consists largely of basement membranes of proximal convoluted tubules. If one of the animal's kidneys be decapsulated and its surface bathed with a solution of phenol red, we may expect the dye to be taken into the blood vessels with which it comes into contact, so into the general circulation, and, in consequence, eliminated by both kidneys. But if the dye is also capable of passing through the walls of the tubules into their lumina, the kidney with which the solution is in contact should eliminate more of the dye than the other. The result shows that it does.

A rabbit was anæsthetized with urethane. The gastro-intestinal tract was excised after ligature of its vessels, and a cannula inserted into each ureter. The left kidney was decapsulated with extreme care, detectable injury to the parenchyma being avoided. This kidney was placed in a glass oncometer. Urine flow was established by intravenous injection of 5 c.c. of 5 per cent. sodium sulphate solution. The rate of urine flow from the two kidneys throughout the experiment was approximately the same. 3 mg. of phenol red were added to 10 c.c. of the animal's

> own blood, which had been drawn at the beginning of the dissection and kept fluid by heparin. This was warmed to 38° C. and introduced into the oncometer so that the entire kidney was immersed. During the following 27 minutes, seven successive samples of urine were collected from each ureter. Comparison of depths of colour in these showed in every case that slightly more dye was present in the urine from the left kidney than from the right.

This result indicates that some phenol red passed into the tubules either by diffusion or secretion. It might be supposed, however, that the dye diffused into the renal tissue to such a depth that it came into contact with afferent arterioles and diffused into the blood flowing in them. Were that true, the arterial blood in a number of glomeruli of the left kidney contained the dye in higher concentration than that of the blood of the general circulation. No way of excluding this alternative explanation occurred to us, and the experiment was not repeated.

2. *Frog's Kidney: Phenol Red applied to the Tubules of one Kidney by Introduction into the Renal Portal Vein.*—It appeared possible to use the renal portal vein of the frog's kidney for the introduction of phenol red into effective contact with cells of the tubules of one kidney, and thus to cause an accumulation of the dye in them in the sense of Marshall; then, having cleared the vessels with saline, to re-establish the arterial circulation and observe whether this kidney will eliminate dye.

> A large frog, *R. catesbiana*, 250 gm., was anæsthetized with urethane. The cœliaco-mesenteric artery was tied and the intestinal tract removed. Cannulas were inserted into the ureters, the renal portal veins and the central stump of the left posterior renal tributary of the posterior vena cava. Each renal portal cannula was connected with a small perfusion reservoir, capacity 10 c.c. Both reservoirs were filled with oxygenated Hamburger's fluid; to that in the left was added phenol red to make 0·3 per cent. The aorta and the posterior vena cava were clamped above the kidney, the clamp on the renal vein tributary removed, and the fluids in the renal portal reservoirs allowed to flow, each at a pressure of 4·5 cm. H_2O. Each fluid, after passing through the kidney with which its reservoir was connected, passed out of the kidney *via* the cannula in the branch of the cava. After the solution had been allowed to flow for 6 minutes, the phenol-red reservoir was emptied, rinsed, and refilled with oxygenated Hamburger's solution. This flowed until the fluid issuing

from the kidney was colourless (3 minutes). Clamps were then placed on the renal portal veins and the renal stump of the posterior cava, and removed from the aorta and the posterior cava proximal to the kidney. Intravenous injection of salt solution was required to inaugurate urine flow.

Urine was collected from each kidney for 30 minutes. Blood, kidneys and urines were analysed for phenol red by the method described by Marshall and Vickers.

Urine from left kidney, 0·022 c.c., contained 0·0009 mg. P.R.
Urine from right kidney, 0·016 c.c., contained 0·0000 mg. P.R.
The left kidney, 0·61 gm., contained 0·018 mg. P.R.
The right kidney, 0·614 gm., contained an unmeasurable trace.
The blood extracts showed a questionable trace of colour.

The experiment was repeated with a similar result.

This experiment seems to show that phenol red deposited in a kidney is later eliminated by it. It suffers, however, from a defect common to all experiments in which the renal portal vein is perfused with the object of excluding the perfusion fluid from the glomerulus. It seemed impossible that 4·5 cm. of water pressure could drive the fluid containing phenol red into the glomerulus through its efferent vessel. Later, however, we actually saw blood corpuscles enter glomerular capillaries through the efferent vessel as the result of pressure in the renal veins of less than 4 cm. H_2O. The experiment is, therefore, open to the objection that during the preliminary perfusion the phenol red may have entered some of the tubules *via* the glomeruli. Its description is retained in this account because the technique may be found useful in other connections.

3. *Perfusion of a Renal Tubule with Saline during Perfusion of the Renal Portal System with Phenol Red.*—In the frog's kidney, it is easily possible to inject fluid through the entire tubule into the capsule of Bowman by way of a cannula inserted into the ureter. Fluid so injected can be recovered after its passage into the capsule of Bowman, according to the technique developed by one of us with Wearn(9). Accordingly, in a number of experiments, after excision of the heart, the ureter was connected with a small reservoir of saline, 18 cm. above the level of the kidney. The renal portal vein was connected with a reservoir of oxygenated Hamburger's solution containing 0·01 per cent. phenol red at 12 cm. The fluid, which was collected by puncture of a capsule of Bowman during the perfusion of phenol red, was distinctly coloured with the dye. It seems certain that the dye had passed through the wall of the tubule.

4. ***Obstruction of the Circulation in Glomeruli; Accumulation of Phenol Red in Tubules arising from them.***—In several experiments, pithed frogs (*R. catesbiana*) were prepared for microscopic observation of the right kidney by transmitted light. A cannula was inserted into the right ureter and connected by slender rubber tubing with a syringe filled with uncoloured saline.

A microscopic field of the kidney surface was selected in which from 8 to 15 glomeruli could be seen, all showing active flow of blood through their capillaries. A map of the field was drawn so that any glomerulus in it could be repeatedly identified.

A glass tube, its end drawn out to a fine tip of about 1 mm. diameter, and this suitably bevelled by grinding on a stone, was mounted in a Barbour stage close to the microscope, in such position that the tip could be pressed against the surface of the kidney in the microscopic field with sufficient force to obstruct all blood flow in the glomeruli included within the circumference of the tip. The obstructed glomeruli were designated on the map and were closely watched throughout the course of the experiment, to be certain that no movement of the stagnant corpuscles within them occurred.

After adjusting the tube as described, phenol red was injected into the anterior abdominal vein. The dosage in one experiment was 12 mg. for a 300-gm. frog. The visible kidney tissue, with the exception of that compressed by the tube, became intensely red; the area under pressure remained blanched. Ten minutes after injection the heart was excised. During the following 10 minutes, many localizations of concentrated colour became conspicuous in the form of curved columns, corresponding apparently to portions of the lumina of tubules. Twenty-seven minutes after injection and 16 minutes after excision of the heart, the compression tube was lifted from the surface of the kidney, and pressure was exerted upon the piston of the syringe connected with the ureter. The streaks of concentrated dye were seen to move, thus proving that the dye was in the lumina of the tubules. Syringe pressure being maintained, the columns of dye were seen to move through many loops of the tubules containing them, until finally they were forced back each into a capsule of Bowman. Not only did the capsules in the uncompressed areas fill with intensely red fluid, but so also did those which had been compressed and so deprived of glomerular circulation.

In the experiment which supplied the figures quoted above, two of the capsules which had been compressed filled with intensely pink fluid, two others became distinctly pink, and one remained colourless. The experiment shows conclusively that tubules which arose from Malpighian bodies, to which the phenol red had had no access, had taken the dye into their lumina; and it appeared there in highly concentrated solution. Repetitions of the experiment gave similar results. It succeeded repeatedly also in kidneys perfused with phenol-red solution *via* the renal portal vein. This experiment is the first of which we have knowledge, in which direct and unambiguous evidence is obtained of the passage of phenol red through the wall of the tubule and of its appearance in concentration in the lumen, under conditions which permit denial of the participation of glomerular function.

5. *Passage of Phenol Red from the Surface of the Kidney into the Tubule.*—A large female bull-frog was pithed; ovaries and intestines excised; cannula in left ureter connected with syringe filled with saline. The left kidney was arranged for microscopic study by transmitted light; the peritoneum covering its ventral surface was dissected free for a short distance from its mesial border. The heart was excised and a little oxygenated Hamburger's fluid allowed to flow through the renal portal vessels in order to clear the tissue of blood and facilitate observation. Five minutes later, aided by the lowest power of the binocular microscope, 0·15 c.c. of 0·3 per cent. phenol-red solution was injected beneath the peritoneum covering the left kidney. The needle did not enter the kidney substance or any of the overlying blood-vessels. The kidney became intensely red. At the end of 25 minutes a few loops suggestive of concentration in lumina were apparent. Pressure on the syringe connected with the ureter caused almost all of the observed glomeruli (about 20) to fill with pink fluid.

In this experiment the kidney was deprived of all circulation; phenol red applied to its surface passed into the tubules. High degrees of concentration of the dye in the tubules were not apparent.

6. *Experiments on the Excised Kidney.*—The preceding experiment showed that phenol red may pass into the tubule of the kidney deprived of its circulation. The perfusion experiments mentioned in section 4 showed that the surviving tubule can concentrate the dye. It therefore seemed probable that the excised frog's kidney, if immersed in oxygenated saline containing phenol red, would take up the dye and concentrate it. This supposition proved to be correct.* When kidneys, freshly excised from a decapitated frog, were

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* In a single experiment made by one of us in Dr. Dale's laboratory at the National Institute for Medical Research, a thin section of freshly excised rabbit's kidney, after immersion in 0·02 per cent. phenol red in oxygenated Locke's solution, was found to contain accumulations of concentrated dye within lumina of tubules analogous to those observed in the excised frog's kidney.

immersed in Hamburger's solution, to which phenol red had been added (0·01–0·03 per cent.), and through which oxygen was bubbling, the dye passed into the tubules, and, in the course of time, these presented an appearance similar to that described in section 4 (Plate 1, fig. 1).

That the dye in the tubules is more concentrated than is the solution in which the kidney is immersed is easily proved by filling a series of glass capillary tubes, drawn to approximately the diameter of a renal tubule, with known solutions of phenol red of various concentrations. By laying them one at a time by the side of the kidney, so that they appear in the same microscopic field with the tubule in question, colour comparison can be made with sufficient accuracy to reveal the order of concentration of the dye within the renal tubule. By this method we have observed concentrations in tubules of excised kidneys as high as 10 times that of the solution in which they were immersed.

It is easy to prove also that the columns of dye are in the lumina of tubules, and that the concentration is not within the cells; for when a syringe containing uncoloured salt solution is connected with the ureter and the piston pressed, the columns of dye are driven backward through the tubules into the capsules of Bowman. Fig. 1 is a photomicrograph of a portion of a kidney which had been immersed in 0·03 per cent. phenol red for five hours; fig. 2 is a photomicrograph of the same portion of the kidney after the columns of dye had been driven into the Bowman capsules. When the column of dye has been forced away from the point at which it accumulated, the vacated portion of the tubule appears colourless.

The level of the tubule at which the concentrated dye collects cannot as yet be stated in terms of its known morphology. All that can now be said is that the level is at a distance of several loops or convolutions from the Malpighian body. When salt solution is injected into the ureter of a kidney in which such concentrations have taken place, one never sees a column of concentrated dye pass directly and immediately into a capsule of Bowman; always it traverses several loops of tubule before making its appearance in the capsule.

The time required for the development of concentrated colour within the tubules varies in different tubules and in different kidneys. Free access of the dye solution to the renal tissue must be an important factor, for the first tubules to show the phenomenon are always those at the extreme lateral border. Later, one sees concentrations in tubules on the surface of and within the thicker parts of the kidney. Concentration in tubules at the lateral border has been observed as early as 15 minutes after immersion. The number of tubules showing it and the intensity of the concentrations increase during the first two to five hours of an immersion. When the kidney is allowed to remain in the solution overnight, the concentrations disappear.

The question which suggested the preceding observations may now be regarded as answered. It is easily possible by several methods to show that the frog's renal tubule is capable of taking phenol red into its lumen through its wall under conditions which exclude the participation of glomerular function. The concentration to which the phenol red in the tubules may attain is significantly higher than that in the solution with which the tubules are bathed. The phenomenon as seen in the excised surviving kidney is apparently a conspicuous example of secretion by cells of the tubule, and it is so easily and uniformly elicited that it lends itself particularly well to further study of the responsible processes.

II.

In efforts to analyse the phenomena disclosed in the previous section, we have used cyanide as an agent for inhibiting or abolishing those properties of the renal tubule which depend upon vitality. A special reason for its choice lay in the fact that it was applied for the same purpose by Starling and Verney in their experiments on the perfused dog's kidney.

1. *Action of Cyanide on the Apparent Secretion of Phenol Red by the Excised Frog's Kidney.*—When the two kidneys of a decapitated frog* are excised, and one, the control, immersed in oxygenated Hamburger's solution containing 0·01–0·03 per cent. phenol red, the other in a portion of the same solution containing, in addition, M/100 KCN, the appearance which develops during succeeding hours is not the same in both. After half an hour, a few tubules along the lateral borders of both show characteristic concentrations of the dye; at the end of an hour, the control shows more than the cyanide kidney, and the difference becomes more marked in the following hour. At the end of four hours many tubules in the control kidney contain concentrated dye, while very few or none are to be seen in the cyanide kidney, those concentrations which first appeared having now vanished.

The immediate difference is more striking if the tubules of the cyanide kidney are filled with colourless M/100 KCN solution by ureteral injection before immersion in the phenol-red cyanide solution. Still more conspicuous is the difference if the kidneys be cut into small pieces before immersion in their respective solutions. Within half an hour the pieces of the control show many loops filled with concentrated dye; the cyanide pieces show none.

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* *R. esculentæ* were used in the experiments described in this part.

It is clear that the processes responsible for the accumulation of concentrated phenol red in the tubules of the excised kidney are abolished by the action of cyanide. The early similarity between the behaviour of the cyanide and the control kidneys in the first of the three experiments described is doubtless due to slow diffusion of KCN into the cells.

2. *Cyanide does not prevent the Entrance of Phenol Red into the Tubules of the Excised Kidney.*—When the excised kidney has been injected *via* its ureter with M/100 KCN and allowed to remain in cyanide-Hamburger-phenol-red solution for an hour, examination with the microscope shows it to be diffusely pink. If, now, the ureteral cannula be connected with a syringe containing uncoloured saline, and an injection made, the fluid with which the capsules of Bowman become distended can be seen to be faintly pink.

In another experiment, cannulas were inserted into the ureters and closed with rubber tubing and clamps. A cannula was inserted into the aorta distal to the renal arteries (pointing toward the heart) and the aorta proximal to the renal arteries tied. This cannula was also closed with rubber tubing and clamp. The kidneys, with vessels and cannulas, were carefully excised and immersed in M/100 cyanide-Hamburger-phenol-red solution for an hour. At the end of that time the preparation was removed, rinsed with saline, and the aortic cannula connected with a perfusion bottle containing uncoloured Hamburger's solution. On allowing this to perfuse the arterial (glomerular) vessels, fluid issued from the ureters which was pink in colour. After the first 0·2 c.c. the ureteral fluid was colourless—strong indication that the dye which first issued from the ureter had been contained in the tubules and was not being extracted from the cells.

3. *Cyanide destroys the Power of the Tubules to retain Concentrated Phenol Red within them.*—If the tubules of a freshly excised frog's kidney are injected *via* the ureter with a strong solution of phenol red (0·3 per cent.), the ureter immediately tied, and the kidney immersed in oxygenated Hamburger's fluid (uncoloured), an appearance develops which resembles that of the excised kidney, uninjected, after immersion for several hours in a dilute phenol-red solution. Examination immediately after the ureteral injection shows uniform distribution of colour throughout the tissue; but presently, within the first hour, the dye becomes localized in such a manner that loops of tubule containing concentrated dye are interspersed with parts of tubules in which the dye seems much less concentrated. This appearance of localization does not change

notably during the second and third hours. The concentration, as well as the colour of the whole kidney, then begins to lessen, and if allowed to remain in the solution overnight, it disappears.

The capacity of certain parts of the surviving tubule to retain concentrated phenol red in its lumen during the first four or five hours after excision is conspicuous. If a simultaneous parallel experiment is made with the companion kidney, adding KCN M/100 to the phenol-red solution injected into the ureter and to the Hamburger's solution in which the kidney is immersed, no localization of dye occurs during the first half-hour; the colour remains diffuse; and, in the succeeding three hours of immersion, the kidney becomes paler and paler until only traces of colour remain. It seems clear that the power of the tubule to retain phenol red within its lumen is abolished by cyanide.

Nothing which we have seen in the experiments thus far recounted compels the belief that the process responsible for the accumulation of concentrated phenol red in portions of the tubule of the excised kidney is one of secretion. We have failed in efforts to see evidence of concentration in the wall of the tubule preceding or associated with accumulation in its lumen. In the work of others we find no direct evidence of the existence of secretory capacity.

On the other hand, an explanation of the facts can be found in a combination of the processes of diffusion and selective re-absorption, and for the existence of both of these we have direct proof. If, in the excised kidney, the cells composing one portion of the tubule permit diffusion of the dye solution through them, while the cells of another portion extrude water from and retain phenol red in the lumen, and if, in addition, we assume the existence of a current of fluid from the diffusion level to the extrusion or re-absorption level, we have an explanation which includes and accounts for all the facts observed. Cyanide, employed to eliminate the vitality of the tubule, does not prevent entrance of phenol red into the lumen: it destroys the power of the tubule to retain phenol red within the lumen and completely interferes with the characteristic concentration of the dye. The "vital" function of the tubule is not related to the entrance of phenol red but to its retention and to the extrusion of water.

We assume that the diffusion of water and dye into the tubule takes place at a different level than that at which water is extruded and dye retained. This assumption is more easily credible than is one which pictures the inward and outward movements as proceeding side by side in the same or adjacent cells. It also accords better with the appearance of loops of tubules containing highly concentrated dye, surrounded by others in which the colour is faint.

The assumption of a current of fluid in the tubule of the excised kidney which transports dye from the diffusion level to the re-absorption level is necessary to account for the quantity of dye which is amassed at the latter level. Evidence that such a current actually exists under the conditions of these experiments is presented in the following paragraph :—

4. *Transport of Dye within the Surviving Tubule, and the Action of Cyanide upon it.*—It has already been stated that when a solution of phenol red is injected into the ureter of the excised kidney, the ureter being immediately tied and the kidney immersed in oxygenated Hamburger's solution, its appearance changes gradually from that of a diffusely, uniformly reddened tissue to one in which the colour is localized in a manner resembling that observed in a kidney which has taken up the dye from a solution in which it was immersed. If, instead of phenol red, a carbon suspension* be similarly injected, the tissue is at first a diffuse and uniform grey ; but after 20 minutes' immersion in oxygenated Hamburger's solution, the carbon is seen to have become localized in a manner roughly resembling the localization of phenol red described above. The experiment is more striking when a colloidal dye is used instead of carbon ; for the latter, in contact with the living kidney tissue, undergoes flocculation to an extent which partially conceals the character of the fluid distribution. Vital red was chosen at the suggestion of Professor Krogh. It was injected into the ureter in 0·4 per cent. solution in saline, the ureter tied and the kidney immersed in oxygenated Hamburger's fluid. Within half an hour the localization of the dye was strikingly similar to that observed in the experiments with phenol red.

In each of the experiments on the localization of phenol red, carbon and vital red following intra-ureteral injection, the companion kidney was injected with the same solution to which KCN had been added (M/100). It was immersed in oxygenated Hamburger's solution containing M/100 KCN. In no case did the localization occur as in the controls. The dye and the carbon remained diffusely and uniformly distributed throughout the tubules.

The factors which are responsible for the currents which these experiments demonstrate have not been investigated. Extrusion of water may possibly create the slight difference in pressure required to move the minute quantities of fluid concerned : the cilia with which certain of the cells of the tubule of the frog's kidney are equipped may be concerned. It is important that the question be studied further.

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* A high dilution of the graphite suspension described by Drinker and Churchill(10) was used.

Specific evidence has been presented in support of each of the assumptions involved in the explanation of concentration of phenol red in the excised kidney, which was advanced on p. 82, with one exception, viz., the active extrusion or re-absorption of water from within the tubule. In addition to a mass of indirect evidence on this point, direct evidence exists. Bieter and Hirschfelder (3) were the first to publish the observation, made on the living frog's kidney, that concentrated masses of phenol red can be seen in the tubule, while dilute dye only appears in the glomerular space. Wearn and Richards (9), in their collections of fluid from the capsules of Bowman, found that the rate at which glomerular fluid can be collected as formed, if multiplied by the number of glomeruli in the kidney, is far higher than the rate at which urine collects in the bladder. Hayman and Richards (11) injected 0·25 per cent. indigo carmine into the intracapsular space and so into the tubule of the living frog: they saw the dye precipitate as it passed down the tubule.

To these observations we wish to add an account of a new experiment which furnishes a very striking demonstration of water absorption from the tubule. It was suggested by the observation made during arterial perfusions of the frog's kidney with dilute phenol red, that one or two loops of tubule in a diffusely coloured microscopic field might be distinguished from all the rest by their content of highly concentrated dye. The explanation which occurred to us was that the perfusion flow through the glomerulus of that tubule had become blocked, and that the consequently stagnant filtrate in the tubule had become concentrated by re-absorption of water. Hence, the following experiment was made :—

The frog's kidney was prepared in the usual way for both arterial and renal portal perfusion, and for microscopic observation by transmitted light. Phenol red (0·02 per cent.) was added to the oxygenated Hamburger's fluid in the arterial reservoir, and perfusion allowed to proceed at pressure of 35–40 cm. H_2O for 10 minutes. Immediately after beginning the perfusion the kidney became diffusely coloured, and its colour did not deepen materially later (fig. 3). At the end of ten minutes arterial perfusion flow was clamped, and perfusion of uncoloured oxygenated Hamburger's fluid begun *via* the renal portal vein at a pressure of 8–10 cm. The whole tissue immediately became paler, because the dye solution was washed out of the blood-vessels. After seven minutes of venous perfusion, individual loops of tubules began to be conspicuous because of the concentration of the phenol-red solution in their lumina. In 15 minutes the entire kidney, in so far as it was accessible to observation, was mottled with curved streaks, each representing the highly concentrated contents of the lumen of a portion of tubule (fig. 4). The phenomenon cannot be caused by secretion: no dye was present in the blood-vessels at the time when the concentrations were occurring, and the cell walls of the tubules were distinctly paler in colour than the lumina during the process. Re-absorption of water, retention of phenol red, and flow of tubular content toward the re-absorption areas are the elements in the only explanation which appears to us to be adequate.

When the experiment was made in identical fashion with the single exception that KCN M/100 was added to the renal portal perfusion fluid, no concentration occurred. The colour of the kidney remained diffuse, becoming paler and paler as venous perfusion continued, until the tissue was washed practically free from colour.

On the evidence as presented, we are obliged to adopt for the phenomenon of the accumulation of concentrated phenol red within the tubules of the excised frog's kidney an explanation which includes diffusion of dilute dye into the tubule, active extrusion of water from the tubule and retention of dye in it, and flow of fluid within the tubule from the diffusion level to the extrusion level. Cyanide abolishes the active extrusion of water, and the power of the tubule to retain the dye at the latter level, and it also stops the intratubular flow. The explanation is applicable also to the perfusion experiments described in this paper, and to the living frog experiments in which glomerular function was eliminated (Part I, section 4); it is to be considered as possibly applicable to any double perfusion experiment with the frog's kidney, in which a diffusible substance, added to the renal portal fluid, appears in the urine.

No claim can be made that these experiments prove the non-existence of a secretory process in the renal tubule. Proof of this might well require the minute examination of every millimetre of the length of the tubule by methods more refined than we now possess. The defensive strength of the secretory theory has long been recognized. The experiments suggest that refuge in the secretion theory should not be taken until the resources of re-absorption have been exhausted.

III.

Belief that it is unnecessary to assume the participation of a secretory factor in the elimination of phenol red by the kidney may be strengthened by the facts which have been presented; and, since phenol red is one of the substances for which that assumption has seemed to others to be most necessary, it may be justifiable to think that such assurance as they convey in relation to the elimination of that substance may extend to that of the normal urinary constituents.

While we regard glomerular elimination and tubular re-absorption as the two great factors in urine formation which are capable of direct and unambiguous demonstration, it is proper to enquire whether the process of diffusion into the tubule, which our experiments have disclosed, is to be regarded as an element in normal kidney function. Before doing so, one qualifying statement should be made. We have tacitly assumed that the process of diffusion of phenol red into the tubule of the cyanide kidney is identical with that in the unpoisoned kidney. We have no proof of such identity; indeed, were we able to test the question, we should rather expect to find readier diffusion into than out of the tubule at the diffusion level in the control kidney, and equal readiness of diffusion in both directions in the cyanide kidney. But the assumption of identity is justifiable and useful, until it shall be proved wrong.

When the excised kidney is immersed in an oxygenated phenol-red solution, the lumen of the tubule contains no dye. The dye at once begins to diffuse in; it is transported to the extrusion level and there concentrated by re-absorption of water. The entire tubular content of dye enters by this process. The same is true in a kidney perfused *via* the aorta with colourless saline and *via* the renal portal system with phenol red (assuming that none of the renal portal fluid reaches the glomeruli).

In the kidney of the living animal, *in situ*, however, after injection of phenol red, we can scarcely think that *concentration* of dye in the fluid within the tubule can be directly increased by inward diffusion of dye: for, by reason of glomerular elimination, that fluid already contains it in concentration as high as or higher than that in which it exists in diffusible form in the blood. Nevertheless it is conceivable that fluid, and dissolved phenol red, may actually be transferred from the blood in tubular capillaries to the lumina of tubules as a result of capillary blood pressure and, in mammals, the osmotic pressure of the concentrated fluid within the tubules. Hayman's(12) estimations of blood pressure in glomerular capillaries in frogs justify the surmise that the pressure within tubule capillaries is high, particularly when the renal arterioles are dilated and aortic blood pressure high. It is problematical whether, in the frog, fluid forced out of capillaries by pressure would enter the tubules ; but this might be expected to occur in those parts of the mammalian tubule which contain concentrated fluid. In the absence of an adequate basis of definite information we can do no more than suggest, as a point of departure for future study, the existence of forces of filtration and diffusion which, by transferring fluid from blood to tubule, may operate as auxiliary to the glomerulus. In this connection it is appropriate to call attention to the important work of Rehberg(13), who, on the basis of a quantitative application of the filtration-re-absorption theory to the problem of urea and chloride excretion in man, has postulated a process of back diffusion, as distinguished from active re-absorption, as an important factor in the return of these substances from the tubule into the blood.

The work of Marshall and his colleagues, which drove them to the belief that secretion is partially concerned in the elimination of phenol red, requires brief comment. They found(5) that only about 40 per cent. of phenol red added to dog's plasma passed through a collodion filter and only 50 per cent. of that added to frog's plasma.(14) They argue that these figures represent the fraction of phenol red in circulating blood which is free to pass through the glomerular membranes. We have made some estimations of the phenol-red content of glomerular fluid, which indicate that in the frog it is nearly all "free." The risk in evaluating the capacities of the glomerular membranes from experiments on collodion is obvious.

They published figures(5) which purport to show that, under certain conditions after injection, more phenol red is eliminated by the kidney in a 5- or 15-minute period than is brought to it free in the blood—the excess, in their view, being secreted from a store previously accumulated in renal cells. In arriving at this conclusion they assume maximal blood flow through the dog's kidney to be 5 c.c. per gram per minute. We believe this figure to be too low. Among the data given by Starling and Verney(6) and Eichholtz and Starling(15) may be found six instances of rates of flow through the perfused dog's kidney of 6 c.c. per gram per minute or over ; one is 7·3 c.c. The manipulations required in the estimation of blood flow through the kidney *in situ* always, we believe, lessen it. Measurements in the rabbit's kidney sometimes amount to between 6 and 7 c.c. Substitution of the figure 7 or 8 for rate of renal blood flow materially alters the force of their argument. For these reasons we do not feel compelled to agree with their conclusions.

The point at issue between Edwards and Marshall on the one hand and Bieter and Hirschfelder on the other is illuminated by our experiments. These show clearly that tubules deprived of their glomerular supply can still acquire and concentrate phenol red and that secretion need not be invoked to explain this. On the other hand, we have found no reason for believing that the glomerulus is not the chief, if not the sole, path of elimination of phenol red under normal conditions.

Starling and Verney chose to apply the secretion theory in explanation of their results on the elimination of phenol red by the perfused dog's kidney. The essential fact which they elicited was that cyanide lessened the concentration of phenol red in the urine and the rate at which it was excreted, while it increased the concentration and rate of excretion of chloride. Hence, in respect of both substances, the urine, after cyanide, had the composition of a glomerular filtrate from the plasma. They argued that cyanide suppressed a secretory process for phenol red: a re-absorption process for chloride.

Starling and Verney's figures show also that cyanide does not cause conspicuous diuresis. In a paper from Starling's laboratory by Gremels and Bodo(16) this fact is used as an argument against the view commonly held that the volume of fluid eliminated in the glomeruli is much greater than that which leaves the kidney as urine. "This filtrate, however, was not, as imagined by Cushney, 80 or 100 times the final amount of fully formed urine, but exceeded the latter little, if at all, in amount." The implication here is that suppression of active re-absorption by cyanide involves interruption of all passage of fluid from the lumen of the tubule into the blood.

We believe these arguments to be misleading. There is no reason for believing that because cyanide abolishes active re-absorption it thereby prevents passive transfer of fluid from the tubule into the blood. Blood flow in capillaries surrounding the tubules was maintained in the cyanide kidney, and the plasma proteins were capable of drawing fluid into the blood by their osmotic pressure. We believe that this force, which Starling long ago demonstrated to be effective in causing the absorption of salt solutions into the blood vessels, is an effective force for causing, in the cyanide kidney, passage of fluid from the tubule into the blood stream: and the failure of cyanide to cause diuresis is to be regarded as evidence that the volume of fluid moved passively in the cyanide kidney may be as great as that actively re-absorbed in the normal kidney.

The remnant of fluid which escapes passive re-absorption in the cyanide kidney and appears as urine must, of necessity, have the same composition as the fluid which entered the tubule from the glomerulus, for the semi-permeability of the tubule for phenol red is abolished by cyanide. Starling and Verney failed to take account both of this fact and of the possibility that active re-absorption in the normal tubule may be replaced by passive re-absorption from the cyanide tubule. When these are considered, it is seen that the observed changes in concentrations and in rates of elimination of both phenol red and chloride, which result from cyanide, are open to one explanation, viz., suppression of active re-absorption and selective semi-permeability in the renal tubule.

The above considerations, to which our cyanide experiments have brought us, are practically identical with those advanced by Dunn and Jones(17) two years ago in explanation of urea retention in experimental oxalate nephritis in rabbits, and a criticism of the conclusions of Starling and Verney, similar to that which is expressed above, is implied in a footnote to Rehberg's paper(18). It appears that the tubule poisoned by cyanide, or that more permanently damaged by oxalate, does indeed exhibit non-selective re-absorption of the glomerular fluid as the result of an "endosmotic stream" into the blood, such as was represented in the hypothesis of Ludwig to be the process taking place in the normal tubule—a conception which has long been recognized as inadequate to account for normal function.

Summary.

Experiments have been described which show :—

1. That phenol red applied to the surface of a decapsulated rabbit's kidney passes into the urine eliminated by it.
2. That a similar result is obtained when the equivalent experiment is made with the frog's kidney.
3. When salt solution is made to flow from the ureter through the tubule to the capsule of Bowman, during perfusion of the renal portal system with phenol-red solution, phenol red can be identified in the saline collected from the capsule.
4. Complete obstruction of circulation through the glomerulus does not prevent phenol red from entering the tubule and becoming concentrated there.
5. When an excised frog's kidney is immersed in oxygenated phenol-red solution, the dye passes into the tubule and becomes concentrated.

These facts, which present the appearance of secretion of phenol red by tubule cells, are best explained by assuming diffusion of water and dye into the tubule at one level, active extrusion of water and retention of dye at another, and a

fluid current within the tubule from the one level to the other. The basis of fact in support of these assumptions is as follows :—

1. Cyanide, employed to abolish vitality of tubule cells, abolishes the power of the excised kidney to concentrate phenol red within its tubules.
2. Cyanide does not prevent entrance of phenol red into the tubule of the excised kidney.
3. Cyanide abolishes the power of the tubule to retain phenol red within it.
4. Cyanide stops the fluid current within the tubule from one level to the other: the existence of such a current was demonstrated by injecting carbon or a colloidal dye into the tubule and noting the progressive changes in its distribution.
5. New proof of re-absorption of water from the tubule is presented.

The possibility of reinforcement of glomerular elimination by a process of diffusion into the tubules is suggested.

Reasons are outlined for disagreeing with recent writers who have adopted the secretion theory in explanation of certain features in the renal behaviour of phenol red.

The experiments described in Part I, sections 1–5, and those on the re-absorption of water in Part II, were made in Philadelphia between January 1 and August 3, 1926: the others were made in Copenhagen during March, 1927. Unfortunately the advantage of Dr. Barnwell's collaboration was limited to the work in Philadelphia. Grateful acknowledgment is made to Prof. Krogh for the facilities of his laboratory and for his counsel; and likewise to Dr. Dale, who very kindly permitted the repetition of a number of the experiments in his laboratory in London.

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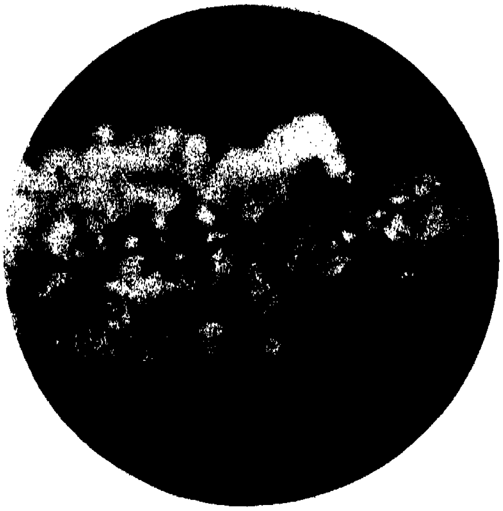

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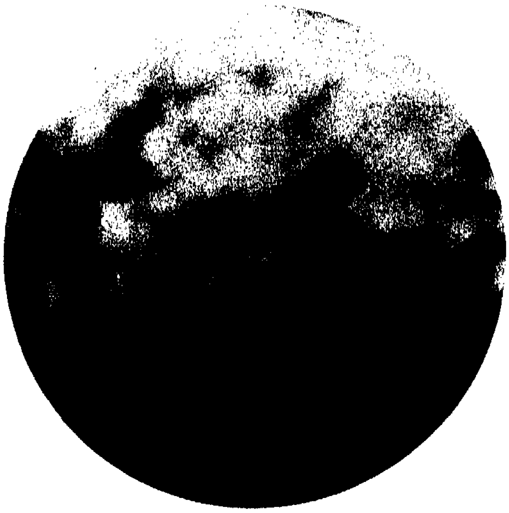

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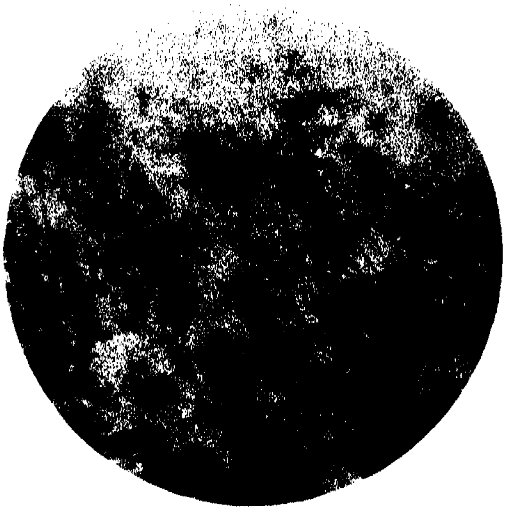

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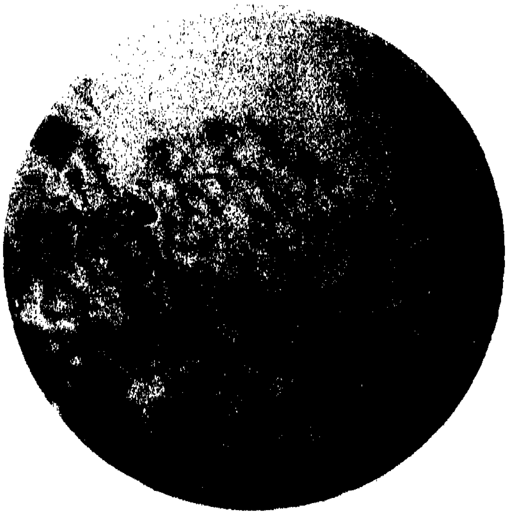

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DESCRIPTION OF PLATE.

PLATE 1.

FIG. 1.—Lateral border of the excised kidney of a small male *R. esculenta*, after immersion for 5 hours in oxygenated Hamburger's solution containing 0·03 per cent. phenol red. Concentrated dye in lumen of tubules. Magnification 29·8.

FIG. 2.—Same kidney as fig. 1 after injection of enough saline into the ureter to force the contents of the tubules into the capsules of Bowman. Oval shadows represent the capsules distended with concentrated dye. Magnification, 29·8.

FIG. 3.—Lateral border of kidney of a large female *esculenta*. Tubules distended with glomerular filtrate containing dilute phenol red as the result of aortic perfusion with 0·02 per cent. phenol red in oxygenated Hamburger's solution at 35 cm. pressure. Magnification, 18·6.

FIG. 4.—Same kidney as in fig. 3, later in the same experiment. Lumina of tubules now contain concentrated dye as the result of perfusing the renal portal system with colourless Hamburger's solution, the aortic phenol-red perfusion having been discontinued. Magnification, 18·6.

The Effect of Gamma Irradiation on Cell Division in Tissue Culture in Vitro.

By R. G. CANTI, M.D., and F. G. SPEAR, M.B.

(Communicated by Sir Frederick Andrewes, F.R.S—Received 27 May, 1927.)

(From the Laboratories of the Research Hospital, Cambridge.)

The experiments described in the following paper were carried out in order to study the quantitative effects of gamma radiation upon mitosis in tissue cultures *in vitro*. They form part of a larger investigation into the causes of the disappearance of certain types of new growth as a result of irradiation.

Strangeways and Oakley* carried out qualitative experiments with X-rays on tissue cultures *in vitro*. They showed that there was a diminution in the number of cells undergoing mitosis. This has subsequently been shown in the case of radium by Canti and Donaldson.†

Quantitative experiments were then undertaken by Strangeways and Hopwood‡ who employed X-rays, and showed that there is a definite drop in the number of mitoses after I *e* unit (Friedrich) has been given.

The objects of the present investigation may be classified under two headings as follows :—

(1) To determine the rate of falling off in mitosis as the duration of exposure to gamma rays increases, the intensity of radiation remaining constant in any one set of determinations.

(2) To determine the relationship of time and intensity which produces the same biological effect, as shown by the decrease in the number of cells in mitosis, by comparing this biological effect with the physical effect produced by irradiation of photographic plates under corresponding conditions.

The expenses in connection with this study were met by a grant from the Medical Research Council, and our thanks are due to the Radium Belge and Messrs. Watson & Sons for the loan of the radium employed.

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* Strangeways, T. S. P., and Oakley, H. E. H. "The Immediate Changes observed in Tissue Cells after Exposure to Soft X-Rays while Growing *in Vitro*," 'Roy. Soc. Proc.,' B, vol. 95, p. 373 (1923.)

† Canti, R. G., and Donaldson, M. "The Effect of Radium on Mitosis *in Vitro*," 'Roy. Soc. Proc.,' B, vol. 100, p. 413 (1926).

‡ Strangeways, T. S. P., and Hopwood, F. L. "The Effect of X-Rays on Mitotic Cell Division in Tissue Culture *in Vitro*," 'Roy. Soc. Proc.,' B, vol. 100, p. 283 (1926).

I.—*The Rate of Falling Off in Mitosis.*

Technique.—The tissue was obtained from the choroid of chick embryos of 6–7 days' incubation. The cultures were grown in fowl plasma and chick embryo extract on a No. 1 coverslip, inverted over a hollow-ground slide sealed with paraffin and incubated at 37° C. Growths of the second sub-culture 18–24 hours old were employed. All cultures were examined under a microscope in an observation incubator kept at 37° C., and no specimens were used which contained less than 20 cells in mitosis.

Exposures were made in a "radium lantern" (*see* fig. 1), which consists essentially of two discs of commercial lead 1 inch in thickness and 4 inches in diameter. Each disc is faced with $\frac{1}{8}$ inch of brass, the centre of which is cut out to receive the hollow-ground slide containing the culture. The two discs are arranged on suitable runners in such a manner that they can be easily approximated.

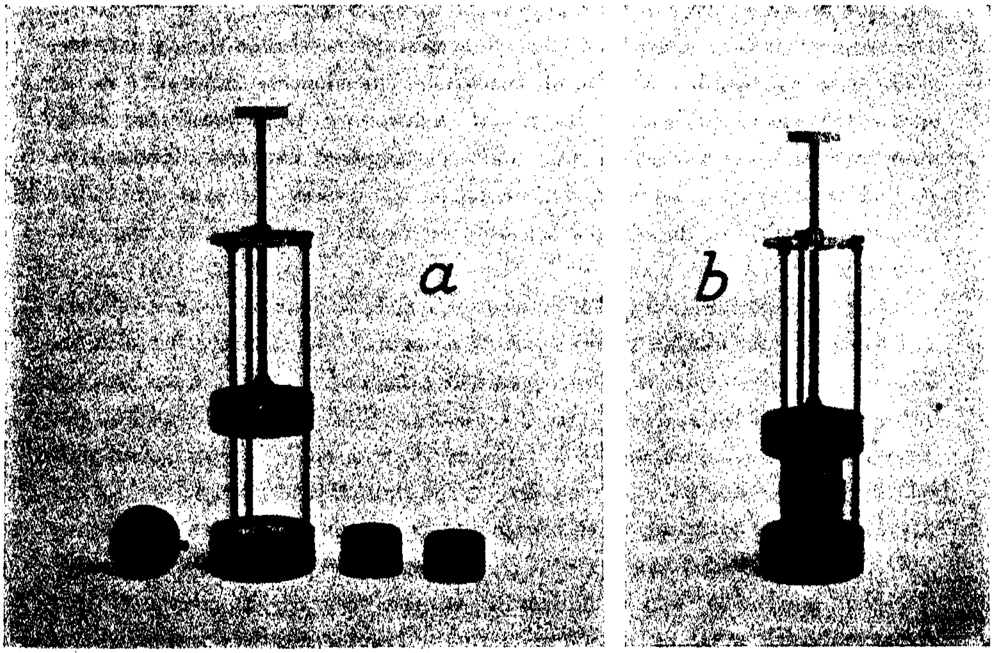

Fig. 1.—Radium lantern.—*a*, showing the container, lead discs on runners, etc., and two tubular distance pieces. *b*, the same assembled.

The radium consisted of 100 milligrams Ra element in the form of the sulphate carried in a brass disc 50 mm. in diameter, which holds the actual container made of platinum. This container has an internal diameter of 13 mm. and is 1·0 mm. in depth with walls 0·5 mm. thick. The radium container is arranged half-way between the two lead discs by means of distancers cut from brass tube 50 mm. internal diameter and 3·5 mm. thick. The object of this design was to standardize conditions of exposure for both the biological and physical experiments, so that they would be independent of the nature and position of surrounding objects.

In order to arrive at any one determination of the reduction in number of the cells in mitosis after irradiation, a number of cultures were selected, half of which were exposed to radium in a special "radium lantern" (*vide supra*), and half of which were fixed and stained to form controls. The reason for using more than one culture for each determination is that, although cultures of the same age and batch show, on the whole, considerable uniformity in the number of cells in mitosis, yet individual variation can be so great that determinations made on a series of single cultures may lead to inconsistent results. By taking, therefore, for each determination the average of figures obtained from several cultures, the margin of possible error can be reduced to a negligible quantity.

Six sets of determinations were made at 0·5, 1, 2, 3, 4 and 5 cm. respectively, and each set was plotted in the form of a curve. The distance between the radium and culture was measured from the centre of the mass of radium salt to the surface of the slide carrying the culture. Whilst in use the radium and lantern were maintained at 37° C. The cultures selected for irradiation were taken from the incubator and exposed to the radium either singly or in pairs. They were then returned to the incubator for 80 minutes,* fixed and stained.

The control cultures were fixed at the time that the irradiation of the corresponding specimens was commenced. In the case of the necessary long exposures at 5 cms. an additional series of control cultures was fixed and stained after the completion of irradiation of the test cultures. The total number of cells in mitosis in the irradiated specimens was then counted and expressed as a percentage of the figure found in the case of the controls.

With the radium acting at 0·5 cm. distance, exposures were made for ½, 1, 1½, 2, 2½, 3, 6, 9, 12, 15, 18, 21, 24, 30, 45 and 60 minutes.

At 1·0 cm. exposures were made for 6, 8, 9, 12, 18, 30 and 60 minutes.

At 2·0 cm. exposures were made for 24, 30, 45 and 60 minutes.

At 3 cm. exposures were made for 50, 140, 210 and 270 minutes.

At 4 cm. exposures were made for 90, 120, 210 and 360 minutes.

At 5 cm. exposures were made at 600 minutes.

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* Strangeways and Oakley, *loc. cit.*

The rate of falling off in mitosis is shown by the counts in Tables I to VI. It will be seen from Table I that with the radium placed at 0·5 cm. from the culture there is no appreciable reduction in the number of cells in mitosis when the exposure has lasted 1½ minutes, but at 2 minutes the fall is marked, *i.e.*, down to 63·5 per cent. of the number in the controls. This fall is continued as the duration of exposure increases until after one hour's irradiation when the percentage is only 0·85. With the radium at 1·0 cm. (see Table No. II) the drop in mitosis does not commence until after the exposure has lasted 6 minutes. Similarly it will be seen from Table No. III that at a distance of 2·0 cm. the fall is delayed until after the culture has been exposed for 24 minutes.

Table I.—Radium at 0·5 cm. from cultures.

Duration of Irradiation in Minutes.	½		1		1½		2		2½		3		6		9	
Number of cells in mitosis	*21*	23	*29*	22	*21*	35	*34*	39	*28*	8	*49*	8	*28*	7	*23*	7
	24	22	*30*	22	—	70	*33*	32	*54*	11	*20*	21	—	12	*42*	11
	22	—	*23*	44	*46*	48	*76*	55	*30*	26	*28*	24	*20*	—	*51*	3
	—	20	*21*	37	*52*	—	*54*	21	*45*	9	*46*	7	*25*	10	*35*	16
	25	26	*43*	—	*36*	35	*54*	23	*22*	13	*21*	13	*34*	10	*42*	8
	20	25	*30*	40	*68*	49	*37*	25	*49*	14	*43*	23	*23*	5	*35*	11
	20	23	—	25	*36*	20	*51*	26	*21*	14	*22*	10	*20*	9	*20*	5
	27	22	*27*	20	*36*	30	*44*	22	*24*	5	*40*	6	*34*	10	*20*	12
Totals	*159*	161	*193*	210	*295*	287	*383*	243	*273*	100	*269*	112	*184*	63	*268*	73
Average per slide	*22·7*	23	*27·6*	30	*42·1*	41	*47·9*	30·4	*34·1*	12·5	*33·6*	14	*26·3*	9	*33·5*	9·1
Per cent.	101·3		108·7		97·3		63·5		36·7		41·2		34·2		27·2	

Duration of Irradiation in Minutes.	12		15		18		21		24		30		45		60	
Number of cells in mitosis	*34*	5	*71*	12	*55*	1	*26*	8	*34*	5	*20*	2	*69*	0	*111*	2
	32	5	*53*	8	*38*	2	*43*	3	*41*	2	*39*	4	*34*	4	*70*	0
	38	10	*50*	7	*41*	4	*39*	2	*24*	2	*37*	1	*30*	2	*32*	0
	33	3	*49*	5	*24*	5	*61*	3	*22*	1	—	1	*21*	0	*44*	0
	117	13	*66*	8	*28*	1	*37*	9	*30*	3	*22*	1	*43*	1	*71*	0
	21	9	*42*	11	*31*	3	*29*	3	*45*	6	*34*	3	*23*	1	*57*	2
	28	8	*49*	11	*46*	0	*44*	1	*20*	4	*30*	—	*40*	0	*59*	0
	22	7	*55*	11	*34*	7	*31*	1	*41*	2	*20*	2	*24*	1	*25*	0
Total	*325*	60	*435*	73	*297*	23	*310*	30	*257*	25	*202*	14	*284*	9	*469*	4
Average per slide	*40·6*	7·5	*54·4*	9·1	*37·1*	2·9	*38·8*	3·8	*32·1*	3·1	*28·9*	2	*35·5*	1·1	*58·6*	0·5
Per cent.	18·5		16·7		7·8		9·8		9·7		6·9		3·1		0·85	

Figures in roman represent number of cells in mitosis in irradiated cultures. Figures in italic represent number of cells in mitosis in control cultures.

Table II.—Radium at 1·0 cm. from cultures.

Duration of Irradiation in Minutes.	6		8		9		12		18		30		60	
Number of cells in mitosis	*25*	21	*58*	19	*37*	15	*36*	24	*27*	10	*31*	5	*56*	4
	28	20	*47*	20	*21*	21	*25*	9	*29*	8	*28*	4	*27*	3
	21	23	*21*	15	*42*	20	*21*	15	*27*	10	*20*	5	—	2
	20	31	*20*	23	*20*	13	*38*	10	*21*	14	*30*	7	*28*	1
	23	20	*31*	22	*27*	21	*32*	9	*20*	7	*23*	5	*43*	3
	20	20	*22*	26	*27*	10	*25*	15	*28*	10	*22*	8	*44*	2
	26	23	*23*	12	*21*	14	*21*	14	*22*	9	*25*	5	*23*	1
	20	26	*40*	16	*45*	15	*29*	21	*20*	16	*23*	6	*23*	3
Totals	*183*	184	*262*	153	*240*	129	*227*	117	*194*	84	*202*	45	*244*	19
Average per slide	*23*	23	*32·8*	19·1	*30*	16·13	*27·1*	14·6	*24·3*	10·5	*25·3*	5·6	*34·9*	2·7
Per cent.		100		58·4		53·7		53·9		43·3		22		7·8

Table III.—Radium at 2·0 cm. from cultures.

Duration of Irradiation in Minutes.	24		30		45	60	
Number of cells in mitosis	**36*	51	*22*	22	20	*36*	10
	66	42	*25*	12	13	*66*	12
	30	53	*38*	11	12	*30*	9
	36	40	*59*	24	10	*36*	11
Totals	*168*	186	*144*	69	55	*168*	42
Average per slide	*48*	46·5	*36*	17·2	13·7	*48*	10·5
Per cent.	—	97	—	47·8	38·2	—	21·9

* Owing to breakage of the coverslip this count was carried out on half the specimen, the necessary correction being made below in the average calculation.

Table IV.—Radium at 3·0 cm.

Duration of Irradiation in Minutes.	50		110		140		210		270	
Number of cells in mitosis	*43*	38	*42*	14	*30*	11	*88*	34	*39*	8
	41	50	*35*	37	*28*	11	*124*	22	*66*	15
	73	80	*47*	21	*26*	11	*82*	25	*35*	5
	54	32	*34*	21	*28*	12	*60*	20	*25*	6
Totals	*211*	200	*158*	93	*112*	45	*354*	101	*165*	34
Average per slide	*52·75*	50	*39·5*	23·25	*28*	11·25	*88·5*	25·25	*41·25*	8·5
Per cent.	—	95	—	58·9	—	40·1	—	28·5	—	20·7

Table V.—Radium at 4·0 cm.

Duration of Irradiation in Minutes.	90		120		210		360	
Number of cells in mitosis	*43*	72	*95*	61	*28*	10	*48*	0
	41	43	*69*	60	*40*	17	*26*	2
	73	48	*91*	64	*21*	11	*56*	3
	54	40	*78*	61	*20*	14	*26*	6
Totals	*211*	203	*333*	246	*109*	52	*156*	17
Average per slide	*52·75*	50·75	*83·25*	61·5	*27·25*	13	*39*	4·25
Per cent.	—	96	—	74	—	48·1	—	10·3

Table VI.—Radium at 5·0 cm.

Duration of Irradiation in Minutes.	600		*
Number of cells in mitosis	*71*	22	*75*
	68	34	*64*
	78	39	*62*
	62	—	—
	31	31	*42*
	38	18	*36*
	59	16	*54*
	55	32	*62*
Totals	*462*	192	*395*
Average per slide	*57·75*	27·43	*56·43*
Per cent.	—	47·5	—

* Owing to the long time occupied in irradiating the test specimens it was thought possible that there might be a falling-off of mitosis in normal cultures. An additional series of Control Cultures were therefore fixed and stained *after* irradiation of the corresponding irradiated specimens, and the number of cells in mitosis in them is shown in the third column.

Again, at 3 cm. (*see* Table IV) no appreciable effect occurred at 50 minutes, but there was a reduction in the number of cells in mitosis to 58·9 per cent. after 110 minutes. At 4 cm. (*see* Table V) there was no effect in 90 minutes, but a fall to 74 per cent. after 120 minutes irradiation. These effects are shown graphically in fig. 2.

It will be noticed from the figures in these tables that there was no evidence that the number of cells in mitosis was increased as a result of irradiation.

II.—*Relationship between Time and Intensity.*

The rate of reduction in the number of cells in mitosis having been studied, the next problem that suggested itself was to ascertain whether the same amount of radiated energy brought about the same biological effect irrespective of the time over which that energy was radiated. For example, if the radium is moved further from the tissue culture to a distance whereby the incident intensity is halved, then will double the time be required to produce the same biological effect? The choice of biological effect which at first sight would seem most desirable, would be the *complete* cessation of mitosis. Reference however to the graphs (fig. 2) shows that this would not yield a sharp end point, and it was therefore decided to choose a corresponding point on each of the curves where the rate of falling-off was rapid and yet the readings sufficiently far removed from those of the controls to be significant. The point at which 50 per cent. fall in the number of cells in mitosis occurred fulfilled those requirements, and a graph (fig. 3) was therefore constructed in which the time to produce this effect was plotted against distance from the radium.

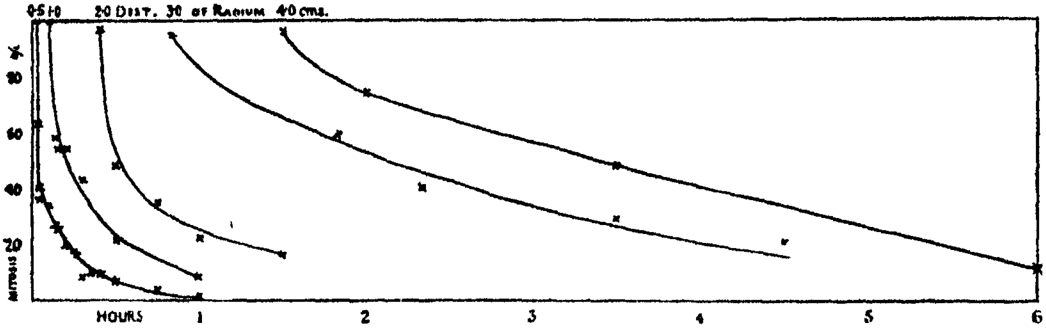


Fig. 2.—Relation between time and intensity. Abscissæ—Irradiation in hours. Ordinates—Mitosis percentage in irradiated specimens compared with control cultures.

The graph thus constructed gives the relationship between time and distance, and the gradual tendency of the curve to become horizontal indicates that under the conditions of the experiment there is a distance beyond which the given biological effect does not occur, however long the radium is allowed to act.

Owing to the fact that the radium was not a point source, and that scattered radiation could not be neglected, it was not possible to undertake a reliable calculation of the intensities used. A means, therefore, of measuring the intensity by direct experiment on photographic plates was devised, and the investigation was undertaken by Prof. F. L. Hopwood and Mr. Thorne Baker.

A series of glass photographic plates of the same size as the microscope slides carrying the tissue cultures was exposed for various times to the radium in the radium lantern at the same distances as in the case of the cultures. The resulting blackening of the plates was measured by a Toy selenium-cell microphotometer. Stringent precautions were taken to obtain plates of uniform emulsion, to give them the uniform development, and to use only those measurements which were found to lie in the region of correct exposure, as shown by a graph of exposures. From these photometric observations it was possible to obtain a curve of equal blackening as a measure of the physical response to irradiation.

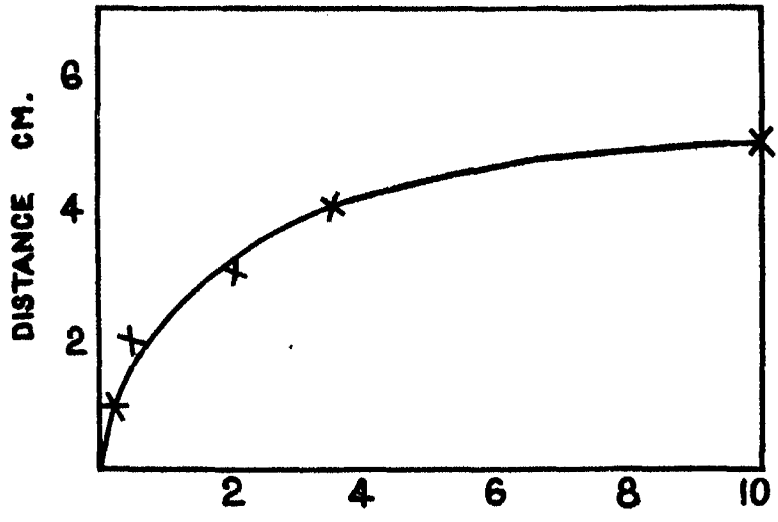


FIG. 3.

Table VII shows results obtained, the reciprocals of the time giving the relative intensities pertaining to those distances.

Table VII.

Cm.	Relative time.
1	1
2	3
3	6
4	10 (interpolated)
5	15
7	20
10	28·5

Times and distances at which equal blackening of the photographic plates occurred.

Fig. 4 shows these readings plotted as a curve in a similar fashion as in the case of the curve of equal biological effect, the physical conditions, except as regards temperature, being the same in both sets of experiments.

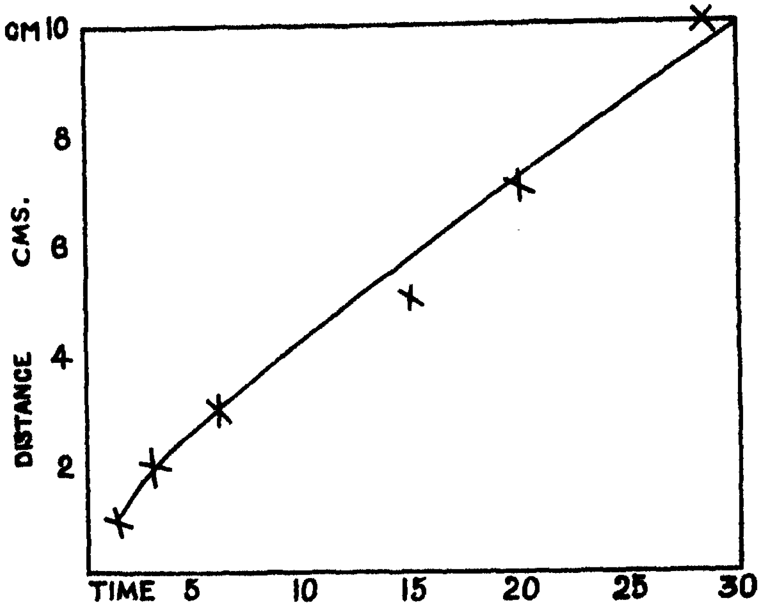


FIG. 4.

In Table VIII the relative intensities at various distances are tabulated with the observed and calculated times necessary to bring about the same biological effect.

Table VIII.—Showing time required to produce a 50 per cent. fall in the number of cells in mitosis.

Distance of Ra from culture. (cm.)	Relative intensity (obtained by photometric observation).	Actual time taken to produce 50 per cent. fall in mitosis.	Time calculated on physical basis $\frac{1}{\text{intensity}} \times 12$.
1	1	12	12
2	$\frac{1}{3}$	30	36
3	$\frac{1}{6}$	125*	72
4	$\frac{1}{10}$*	210	120
5	$\frac{1}{15}$	600	180

* Interpolated.

A comparison, both of the two curves and of the third and fourth columns in the table, shows the difference between the physical response of the photographic plate and the biological response of the cultures. It indicates that the action of radium is not purely cumulative, but that as the intensity is decreased a longer time is required to bring about the same biological effect than would be anticipated by purely physical laws.

Conclusions.

1. Gamma radiation has a definite effect in reducing the number of cells in mitosis in tissue cultures *in vitro.*

2. This effect is not demonstrable until the culture has been subjected to irradiation for a period of time, which is greater with a smaller intensity of irradiation. When this threshold is passed the effect upon mitosis is sudden and well marked.

3. No evidence is seen of any stimulating effect of gamma rays on cell division under the conditions of the experiments.

4. Under the conditions of the experiment a longer time is required with the weaker intensities to being about a biological effect than would be anticipated from purely physical considerations.

5. The action of radium is not purely cumulative, and there is a minimum threshold of intensity, below which a given biological effect will not take place, however long the time of exposure.

The Distribution of Lymphatics defined by Fatty Acid Compounds developed in the Autolysis of their Contents.

By J. Lorrain Smith, M.D., F.R.S., and Theodore Rettie, D.Sc.

(Received June 17, 1927.)

(From the Department of Pathology, University of Edinburgh.)

[Plate 2.]

During the course of our study of the early phases of fatty degeneration, we had occasion to consider the evidence advanced by some investigators in favour of the view that the tissue changes due to autolysis after death are continuous with, and in some respects identical with, those of pathological degeneration during life. While we were, for this purpose, examining sections of the liver by microscopic and microchemical methods, our attention was arrested by the evidence of lipid autolysis occurring in the portal lymphatics of certain animals. After death, autolysis slowly proceeds in all tissues and fluids of the animal body, and chemical changes in proteins and fats or lipids result. Our investigation was restricted to changes in fats and lipids. Animal tissues and fluids kept for a few days at room temperature, all exhibit, under the polarising microscope, doubly refractile globules showing a black cross on a light ground. On the other hand, these form in some tissues earlier than in others. They result from autolytic hydrolysis of fats and lipids, leading to the formation of spherules of compound soaps.

Of all the organs, the liver is one of the most suitable for the study of the process of autolytic saponification, and if that of the ox, or of smaller animals such as the guinea-pig, be used, it will be found that in certain cases, though not in all, the globules of soap may be observed in the portal lymphatics before they appear in the epithelial cells, the blood, or any other site in the liver. In such cases they fill the channels continuously and, like an artificial injection, give definition of the form and distribution of the lymphatics. In a section under a polarising microscope, the lymphatics stand out as structures of stem and branch brilliantly lit on a dark ground; under an ordinary microscope with transmitted light, they are opaque, almost black, on a relatively clear ground. With the same microscope when the mirror is turned off and only reflected light is available, they appear grey-white. With either transmitted or reflected light the anatomical form and arrangement of the portal lymphatics are well brought out by the ordinary binocular dissecting microscope with a magnification of 30 diameters.

In the same conditions of autolysis the lymphatics of other organs also—pancreas, kidney, mesentery, lungs, meninges—are similarly defined. The liver of the guinea-pig, however, may well be selected for investigation because the portal lymphatics are very abundant and their contents rich in saponifiable material.

With autolysis of the tissues there is, before long, an accompanying growth of bacteria and moulds. In tissues, however, in the condition most favourable for observation the autolytic changes are well defined before these organisms have grown to any extent. The original observations which led us to begin this investigation were made on a piece of calf's liver, as it came from the butcher's shop.

The body of the guinea-pig to be examined should not be opened until the process of autolysis has developed sufficiently for the purpose of the observations. Tissues exposed to the air, and especially in summer weather, are soon invaded by moulds, and we have found that where growth is abundant the formation of doubly refractile globules is relatively absent. It is possible to control the growth of moulds and bacteria in an organ removed from the body, by keeping it in an antiseptic atmosphere, and for this purpose toluol vapour is recommended. Toluol, however, has a distinct inhibitory effect on autolysis of lipids, and in the surface layers directly exposed to the action of toluol there will be little, if any, formation of the globules.

We tried the method of immersing the guinea-pig's liver in toluol. The growth of organisms was prevented, but autolytic globules were found only in the centre of the organ away from the direct action of the toluol. We have not discovered an antiseptic which inhibits the growth of micro-organisms without interfering with the process of autolysis.

The method of observation is as follows: Of tissue from a liver kept for a few days—seven or more—sections are cut by hand with a sharp razor, spread on a slide, and cleared in glycerine. To this direct method there is an alternative; since the saponified material is very insoluble in fat solvents, the tissue to be examined may be fixed in spirit, the sections then made by hand, as before, dehydrated in alcohol, cleared in clove oil, and mounted in balsam. Of aqueous fixatives, saturated corrosive sublimate is the only one we have found, so far, that does not disperse the globules.

The soap globules have the following characteristics: They are insoluble in alcohol, ether and chloroform, opaque in transmitted light, and doubly refractile in polarised light, as already stated; they stain black with osmic acid, dull red with Sudan; they are rapidly hydrolysed and dissolved in dilute alkaline solutions; in dilute acids they lose their double refraction and are partly dissolved.

Fig. 1 (Plate 2) is a photograph of a section of the liver of a guinea-pig, after autolysis for 10 days, no fixation, cut by hand, mounted in glycerine. For this preparation a section was made parallel to the surface of the liver to show the distribution of the lymphatics, as they appear underneath the outer serous capsule. The photograph shows acini of the liver; confined to the region of the portal tracts and forming an outline to these acini, strands of lymph capillaries, partly a meshwork and partly free terminals, not penetrating among the columns of liver cells, and not coextensive with the blood sinusoids.

With regard to the distribution of the portal lymphatics, the results brought out by this method are in agreement with those of Herring and Simpson (1), obtained by injection.

Fig. 2 is a photograph by polarised light with crossed nicols; section from liver of guinea-pig, autolysis for eight days, tissue not fixed, section by hand, glycerine mount. The normal arrangement of the lymphatic channels in relation to the acini has been a little disturbed in the process of mounting. Saponification has occurred, as yet chiefly in the contents of the lymphatic channels; only a few doubly refractile globules appear in the acinar tissue occupying the areas between the lymphatic branches.

This preparation demonstrates the beginnings of the lymphatics in the form of closed tubes, the rich content of saponified material in the channels, and the restricted distribution of lymphatic capillaries in the liver, where blood capillaries or sinusoids are everywhere present in abundance.

To obtain tissues showing the changes due to prolonged autolysis, the body of a dog was kept in the ice chamber for three months. The liver showed abundant doubly refractile globules in the acini, but scattered irregularly and not giving definition to the columns of epithelial cells or the blood channels. They occurred also in the lymphatics, and so massed as to give anatomical definition.

Fig. 3.—Photograph by polarised light of section of liver of guinea-pig after autolysis for seven days. No fixation; glycerine mount. In the centre of the field is a dark shadow due to a blood-vessel; lumen filled with red blood. No doubly refractile globules have yet formed in the lumen of the blood-vessel. Encircling the blood-vessel are lymphatics; branching structures white from their content of doubly refractile spherules.

Fig. 4.—Photograph by transmitted light of section of the liver of a fœtal guinea-pig. Autolysis for 14 days. Fixed in spirit; mounted in balsam. The lymphatics in this liver also are defined by their doubly refractile contents. As yet no sign of saponification in the acini. The interest of the observation lies in the fact that the whole of the fatty material brought to the fœtal liver has come from the maternal blood. In the placenta, however, there was no evidence of autolytic saponification. The photograph shows further that the contents of the lymphatics have saponified before any similar change has occurred in the epithelial cells. The order of saponification frequently observed in the adult occurs also in the fœtal liver.

To interpret the order of saponification involves a distinction in the material to be saponified, and the cases accordingly fall into groups. The first group consists of those in which autolysis in the lymphatics is prior to that in any other site. Another group includes those in which saponification occurs in and among the epithelial cells and in the lymphatics simultaneously; and there is a third group in which it occurs in the acini, but not in the lymphatics. The origin of the material is twofold: (*a*) the lipid of cell cytoplasm is a constant source, and, though it may be sooner or later, evidence of saponification in cells is invariably forthcoming; with the cells may be grouped blood and tissue fluid; (*b*) the masked substance in the lymph, present or absent according to the activity of the lymphatics in the area examined.

In general, our observations confirm the conclusion that portal capillaries contain a lymph of high concentration.

We have, so far, described the material saponified in the lymphatics as a masked constituent of the circulating lymph. The question arises, could not this material have its origin in the endothelium of the wall of the lymphatic and be due to autolysis of cells and not of normal constituents of lymph? While some of the material may have come from endothelial cells, it seems doubtful if more than a small part of it could have this source. We are reminded of Tait and Hewitt's (2) observations on the lipid contained in the endothelium scraped from the intima of the bullock's aorta. At the same time, relative paucity of doubly refractile globules in the blood-vessels is the rule in the conditions we have observed. They may be, and often are, practically absent from the blood-vessels in liver sections, in which the epithelial cells are studded with globules and the lymphatics stand out as if they had been injected with anisotropic material. In relation to this question we made observations on the autolysis of the wall of the lymphatics in a liver when disintegration had reached an advanced stage.

In the liver of a guinea-pig kept until the tissue had been reduced by autolysis to a semi-fluid mass, we found it still possible to demonstrate the portal lymphatics, and not only so but under a dissecting microscope we could, by the aid of a needle, drag out from this mass groups of lymphatics more or less intact, spread them on a slide, mount and preserve them.

Fig. 5 (Plate 2) is a photograph of a specimen obtained in this way. The loosened or fluid liver tissue has been separated from the lymphatics even to the finest terminals, and round them there remains adherent only a little homogeneous brown substance, hardly to be made out in the photograph. Against this degree of destructive autolysis the wall of the lymphatic has been resistant, and it is doubtful if any other element in the texture of the liver has the same power of survival. The prolonged resistance to autolysis by the wall is in marked contrast with the appearance of autolytic globules in the lumen, always an early change when it does occur, and sometimes prior to any other.

There is therefore no evidence that autolysis of the wall is the source of a substantial part of the material which is found in the lumen of the lymphatic channel.

Further proof that the doubly refractile spherules are derived from lymph is obtained on examining a section of a regional lymphatic gland.

Fig. 6 is a photograph by transmitted light of a section of a lymph gland from the hilum of an ox liver; tissue fixed in spirit after autolysis for 10 days; cut by hand, mounted in balsam. It shows the peripheral sinus immediately under the capsule at a point where it is filled by a mass of opaque spherules. Since they are doubly refractile, they light up under the polarising microscope. Throughout the channels, therefore, from the minute terminals to the sinuses of the regional glands, the lymph shows the same changes.

The general result of the investigation is a demonstration of the form and arrangement of the portal lymphatics of the liver. The anatomy of the lymphatics is revealed by a process of auto-injection. The interpretation of the process raises questions regarding the production of lymph in the liver and the composition of the lipid it contains. The method takes advantage of spontaneous hydrolysis of the normal contents in the lymphatics after death. As neither fixation nor staining are necessary, the sole effect in virtue of which the lymphatics are defined is due to agencies present in the tissues. The hydrolysis is the direct result of cessation of the circulation of blood and lymph in the liver. Lipid is present in the plasma of the sinusoid blood, in the tissue fluid of the acinus, in the cytoplasm of the epithelial cells, and in the lymph. Sooner or later autolysis of lipid will occur in each of these sites, but according to our observations the channel of the lymphatic has proved, in many cases, a site of autolysis more favourable than any other in the liver. In other words, with the absorption of the lipid from the tissue fluid into the channel of the lymphatics there is associated some condition responsible for the relatively rapid development of autolytic change. Of the four sites, the lymphatic channel, though the last in the physiological sequence, is often the first to show the hydrolytic changes. Further investigation of the order of autolysis would probably throw some light on the relation of the liver to fat metabolism and transference.

Another question is the nature and relative amount of fatty substance in the lymphatics of the liver. Analyses of lymph have shown that in regard to fat content it does not widely differ from blood plasma. Apart from the lymph of the lacteals, which may be creamy from the mass of absorbed fat, the lymph of the liver is more concentrated than any other in the body. According to Starling (3), the lymph of the portal capillaries contains 6–8 per cent. proteids, and is, " in fact, almost as concentrated as the plasma within the blood-vessels." While the observations we have made lead us to conclude that the lymph of the liver, as a rule, is correspondingly rich in lipid also, it must be remembered that in certain cases the portal lymphatics failed to show autolytic spherules. The variation in lipid content may depend on the metabolic activity of the liver in relation to the fat of the diet, or to some more specific condition of metabolism. We have recorded observations on the liver of a fœtal guinea-pig to show that the same hydrolysis of lipid occurs in the portal lymph in the absence of immediate absorption of fat from the alimentary canal.

From these observations we conclude that normal lymph of the liver contains in masked invisible form simple or combined fat, and that in the course of a few days after death this undergoes hydrolysis in the channels. The fatty acids set free form insoluble soaps.

It is sufficient for the present purpose to follow the accepted doctrine, and state the reaction simply as a combination of fatty acid and base. On the other hand, we do not yet know in what form the fat exists in the lymph. The masking of it may be due to a division so fine that it is invisible, or it is invisible because it is in a form of combination which fat stains do not detect. Until we have information on this, it will be impossible to describe the steps in the process of saponification more exactly. That it is a process in which the form of the soap is gradually changed seems to us probable from observations on the temperature of the clearing point and the solubility in various media.

The foregoing account of the lymphatic system is a record of observations made, for the most part, on tissues (liver, etc.) from the ox and the guinea-pig. In them we found it relatively easy to obtain evidence of lymph autolysis. For confirmation, however, the tissues of other animals were examined, including sheep, pig, dog, cat, rabbit, rat, mouse, chicken, and in each positive results were forthcoming. At the same time, there are interesting variations even in the same species.

To these are to be added observations on human lymphatics, chiefly with regard to portal tracts. In subjects a few months old there is evidence of lymph autolysis similar to that already described in animals. As age advances, the evidence, for reasons not yet determined, becomes less definite. With the widespread and abundant formation of autolytic globules in the epithelial cells of adult human liver, it becomes more difficult to define the lymphatic structures; there is no massive content of spherules to give them prominence. It will be of interest to inquire to what extent, if at all, this difference between infant and adult is related to the changes that normally occur in the composition of the fat as age advances.

The definition of lymphatics by this method has opened up a number of questions regarding the circulation of lymph. There are, for example, indications that lymphatic glands have, as indeed has long been suspected, some definite metabolic activity in relation to the current. So far as lipids are concerned, the method will enable us to observe the changes *in situ*.

In the same way, with the method comes the opportunity of observing the activity of blood capillaries in the absorption of fat from adipose tissue.

In general, the method is applicable to the lymph or blood circulation of any area or tissue specially concerned with the metabolism of fat.

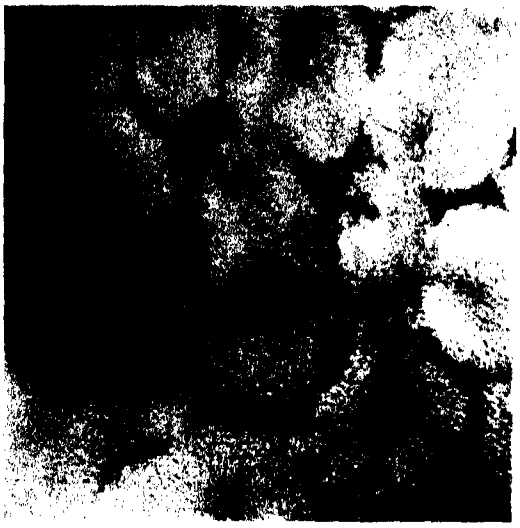
No attempt has been made to investigate œdema and other pathological conditions of the lymphatic circulation, or the effect on lymph of metabolic disturbances, such as fatty degeneration of the liver. While we have every reason to believe that the application of the method to such problems will be fruitful, we have been compelled to limit ourselves, for the present, to the field we have described.

CONCLUSIONS.

The following are the chief subjects discussed in the paper :—

1. A method of demonstrating the anatomical form and arrangement of the lymphatics of the liver by observing the *post-mortem* autolytic changes in the lymph.

2. Autolysis of the lipid contained in this lymph forms doubly refractile



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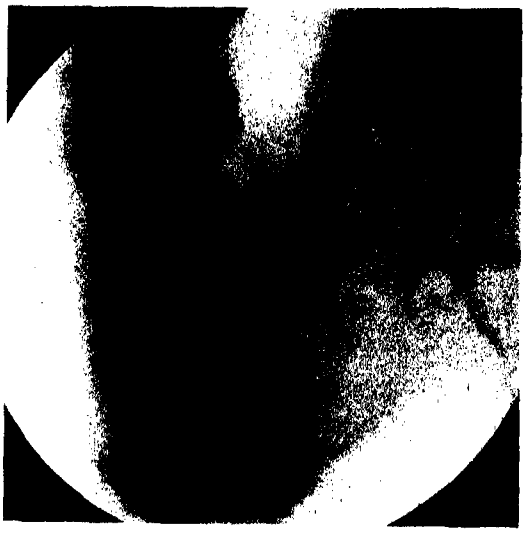

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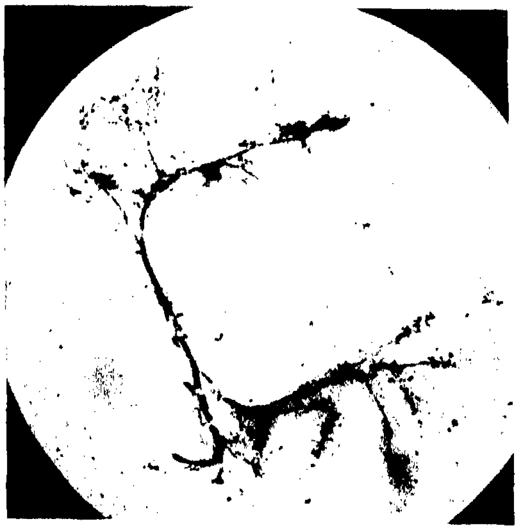

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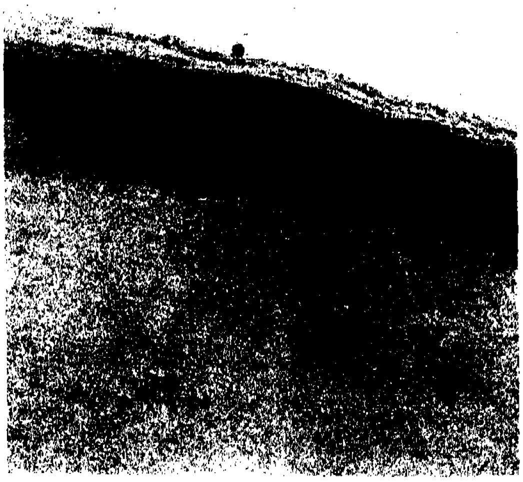

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globules of "soap," often in such quantity that the lumen of the channel is filled continuously.

3. Lymphatics of the liver are defined because in them the globules form abundantly and at a stage when, as yet, no sign of them is to be found in any other site.

4. The portal lymphatics of the guinea-pig's liver afford the best demonstration of lipid autolysis in lymph. With the description of their structure is included a preliminary discussion of some of the physiological questions which arise in applying the method.

We beg to acknowledge, with thanks, the helpful criticism we have received from colleagues and fellow workers. The preparation of the photographs has been Mr. Richard Muir's contribution to the investigation, and we are much indebted to him for his generous assistance.

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DESCRIPTION OF PLATE 2.

FIG. 1. Liver of Guinea-pig. × 30. (See p. 104.)
FIG. 2. Liver of Guinea-pig, polarised light. × 30. (See p. 104.)
FIG. 3. Liver of Guinea-pig, polarised light. × 40. (See p. 104.)
FIG. 4. Liver of Guinea-pig Fœtus. × 30. (See p. 105.)
FIG. 5. Lymphatic structure detached from autolysed liver of Guinea-pig. × 18. (See p. 106.)
FIG. 6. Lymph Gland of Ox. × 40. (See p. 106.)

The Mammalian Lacteal: its Histological Structure in Relation to its Physiological Properties.

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(Communicated by Sir Charles Sherrington—Received July 6, 1927.)

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(Plates 3–5.)

1. *Introduction.*

One of us (H. W. F.) (7, 8) has already noted that the mesenteric lacteals contract on appropriate stimulation in a number of animals. He also observed in the guinea-pig and the rat that the vessels were rhythmically contractile. In the latter species the nuclei of circular muscle fibres in the lacteal wall could be seen in the living condition. The difficulties, however, of making out the part played by smooth muscle cells and nuclei in such contractions are very great in the living preparation. It was, therefore, resolved to supplement these observations on the living mesentery by others on fixed and stained material. In some cases the excised lacteal had actually been observed to contract in the living mesentery.

The mesenteric lacteals of the cat have been taken as a type, and special attention has been paid to the distribution of smooth muscle fibres in the lymphatics in the various species studied.

2. *Methods.*

An apparatus enabling microscopic observation to be made on lacteals in the living animal has already been described by Florey elsewhere (7). For the preparation of fixed specimens, areas of mesentery containing lacteals were lightly stretched over a wide glass tube; a thread was tied round the tube; the mesentery outside the thread was cut away; the tube and the stretched portion of mesentery were then fixed in 2·5 per cent. or 5 per cent. formalin. Another method of obtaining uncurled pieces of mesentery was to place a piece of filter paper beneath the mesentery; a portion of the latter containing lymphatics was then cut out with the underlying filter paper, which was then dropped, mesentery downwards, into a Petri dish of the fixative. The great majority of mesenteries so treated remained lightly adherent during fixation to the paper. They were easily detached prior to staining.

A number of stains were used, but the most suitable general stains for whole preparations were found to be the iron hæmatoxylins of Heidenhain and of Weigert. Both these methods, after careful differentiation beneath a staining microscope, give good pictures of lacteals.

Whole preparations made in the ways referred to above can be studied with powers up to a 3, and even a 2 mm., apochromatic oil-immersion objective. Sections were prepared by fixing pieces of mesentery in 5 per cent. formalin, cutting at 5μ, and staining by the usual methods.

3. *Histology of the Lacteals.*

A. *The lacteals of the cat.*—Sections of mesenteric lacteals show :—

(i) An endothelial layer. The cells comprising this are very similar to the endothelium of a vein, except that their arrangement and outlines are more irregular. Often the nuclei of the lacteal endothelium are rather obliquely set (*see* Plate 3, fig. 4). This, of course, is the result of the more irregular imbrication of the endothelial cells in lymphatics.

(ii) An adventitial layer. In a fairly large lacteal (see Plate 4, fig. 6), of 150 by 450 μ in diameter, the wall of the lacteal is made up of fine collagen fibres. Between these are small and rather darkly staining nuclei which we identify as connective tissue nuclei. The outermost fibres of the adventitia merge into those of the mesentery.

An elastic framework, mingled with the collagen fibres, is constant in lacteals other than lymphatic capillaries. These fibres are mainly longitudinal with frequent cross-anastomoses. The latter are often cut through obliquely in transverse sections of the vessel (see Plate 4, fig. 7).

The collagen fibres of a lacteal (Plate 4, fig. 6) are very thin as compared with those of a vein of approximately the same calibre (Plate 4, fig. 9). When the vessel contains lymph at the moment of fixation, the proteid is precipitated to form a very fine granular coagulum. Two small lacteals, closely applied against the larger vessel, are also shown in fig. 6 ; their walls are composed of endothelium and a few very fine collagen fibrils. Capillary blood-vessels are often found closely applied to the lacteal's wall.

The above description merely confirms that given in the well-known works on microscopic anatomy [*e.g.*, Schafer (14), Delamarre in Poirier (11), Prenant and Bouin (12) ; Bartels in Bardeleben (1)].

Smooth muscle fibres, when they occur, are intimately mingled with the collagen fibres and the connective-tissue cells of the lacteal wall. Some care is necessary to distinguish between smooth muscle and the other elements, and we have found the following criteria valuable :—

(i) The nucleus of the smooth muscle fibre is relatively larger than that of the connective tissue cell.

(ii) Its tendency to take up stains is generally less marked in one and the same preparation.

(iii) The smooth muscle nucleus is transversely or rather obliquely orientated with regard to the long axis of the lacteal. It is never parallel to the latter. This is in contrast with the endothelial nuclei.

(iv) The spiral nuclear fold of the smooth muscle nucleus is often a very useful criterion in fixed and stained specimens. With certain metallic impregnation methods, such as the Cajal method for the Golgi bodies, this spiral structure is deeply impregnated, as shown by Rio Hortega (13), and confirmed by one of us [H.M.C.; 2]. It then looks like a spiral band wrapped around the nucleus. In carefully differentiated sections stained in Heidenhain's iron hæmatoxylin, the spiral " band " is seen really to be a rather irregular fold or incision running spirally along the nuclear membrane of the smooth muscle fibre (Champy and Carleton) (3). It can also be observed in unstained preparations mounted in a medium of low refractive index.

We have made use of this structure to distinguish between the nuclei of smooth muscle, and, for example, those of connective tissue. We have never seen this spiral incision in cells other than smooth muscle cells; on the other hand, not all smooth muscle nuclei show it. So that, while a nucleus showing spiral folding can be branded as belonging to a smooth muscle fibre, a nucleus in which this structure is not present cannot, on this score alone, be eliminated from smooth muscle. It is necessary, in such cases, to assess the type of cell on points i, ii, and iii also.

Regarding the nature of the spiral nuclear fold, it is quite possibly an artefact, in the sense that it represents a contortion due to abnormal contraction of the muscle fibre when brought into contact with the fixative.

We must point out that the identification of an isolated smooth muscle fibre, lying between collagen fibres and connective-tissue nuclei, cannot be established by staining reactions only. For instance, although van Gieson's stain will selectively stain the collagen fibres red, an isolated smooth muscle fibre will not be stained yellow. It will tend rather to stain like the surrounding (fuchsinophil) collagen elements.

Examination of many sections and whole preparations has led us to conclude as follows: The medium-sized lacteals (*i.e.*, of 100 to 200μ in diameter) contain typical smooth muscle nuclei. In transverse sections of lacteals they are difficult to find. This is because they are relatively few, as compared with the endothelial and connective-tissue nuclei. In longitudinal sections which have shaved the outer wall of the vessel smooth muscle nuclei are more easily found. In whole preparations they can be identified with facility when present (see Plate 3; figs. 2 and 3).

The spiral nuclear folds are shown in fig. 3; in fig. 2 the region of the lacteal depicted does not show these spiral incisions—a rare condition. But even here the orientation, size and position of these nuclei in the connective-tissue framework of the vessel wall enables one to identify them.

In the smaller lacteals smooth muscle nuclei cannot be seen. In Plate 3, fig. 1, is shown a segment of such a vessel some 30 μ in diameter. The endothelial nuclei on the upper and lower surfaces can be seen; a few connective-tissue cells are scantily grouped along the vessel. But there are no smooth muscle elements, nor can any be seen after careful examination of the entire length (4 mm.) of the vessel included in the fragment of mesentery. Yet lacteals of this size in the cat contract on strong stimulation by a faradic current.

B. *The lacteals of the squirrel.*—The vessels in this animal may easily be seen to contract on stimulation by a faradic current applied by means of a fine unipolar electrode. An annular contraction appears at the point stimulated, slightly spreading along the vessel. The diameter of the latter may be reduced by about one-third.

The general histology of the lacteals is much as in the cat. Extensive search for smooth muscle nuclei or fibres in small and medium-sized lacteals (*i.e.*, in vessels up to 300 μ in diameter) has convinced us that muscular elements are nearly—if not entirely—absent. We say " nearly absent " although we have not seen any smooth muscle nuclei in vessels up to the limit mentioned (300 μ diameter). But obviously the total length of lacteal examined beneath the microscope is far inferior to that in a whole mesentery. Still stronger evidence, however, that contraction in these vessels may be independent of muscle, is furnished by the excision and careful examination of segments of lacteal which had previously been seen to contract in the living. Examination of such segments, each over 4 mm. in length, has failed to reveal smooth muscle nuclei or fibres.

In Plate 3, fig. 4, one side of a lacteal from the squirrel's mesentery is shown. The diameter of this vessel, in a fixed and stained whole preparation, is 110 μ; its length, 11·25 mm. No smooth muscle nuclei or fibres can be found. The slightly oblique and pale nuclei (E.2) are seen, on focussing, to be obviously endothelial. The darkly stained and sometimes oblique nuclei (X in the figure) are easily identified on focussing, with the nuclei of the fat cells and their connective tissue framework lying about the lacteal.

C. *The distribution of smooth muscle nuclei and fibres in some other animals.—In the guinea-pig, the rat and the mouse,* the medium-sized lacteals contain abundant smooth muscle. In the rat smooth muscle nuclei and fibres are also found in fairly small vessels. Thus, in Plate 4, fig. 5, there are abundant smooth muscle nuclei, the majority of which show the spiral folding. The outline of a muscle fibre (rarely seen in whole preparations) is shown at M.F. in this figure. The diameter of this vessel was 50 μ.

In some specimens of lacteal of the *dog*, the *hedgehog*, and the *pig*, smooth muscle was plentiful in the larger vessels, particularly in the dog. Our scanty preparations of these species show no small lacteals, so we cannot say whether these contain muscle or not.

In the *rabbit* smooth muscle nuclei are easily identified.

(4) *The Nerves of the Lacteals.*

Observations were made on the nerves of these vessels by vital staining with methylene blue. The dye used was "methylen blau, rectif, nach Ehrlich of Grübler," and was administered in warm saline intraperitoneally.

In the guinea-pig the nerves of the lacteals were very clearly shown after injections of 0·5 per cent. and 0·25 per cent. solutions of the dye. The amount injected varied from 25 c.c. to 75 c.c. The animals were killed 1½ hours after the injection. Pieces of mesentery, stretched out on filter paper, were fixed in a saturated aqueous solution of ammonium picrate. They were mounted, examined and photographed also in this medium.

The nerves of the lacteal wall pursue a mainly longitudinal course and are finely varicose (Plate 5, fig. 10). They are amyelinated. No nerve cells are visible.

In the squirrel (Plate 5, figs. 11 and 12) the peri-lymphatic nervous network was demonstrated by an intraperitoneal injection of 0·5 per cent. methylene blue, and the animal was killed 1 hour afterwards.

In the large lacteals (*i.e.*, in those of 200 μ in diameter and above) the nerve fibres anastomose freely and form a network. Between the anastomoses nerve cells can sometimes be seen. Fine twigs can also be observed to run towards, and to touch, the smooth muscle fibres which exist in the large lacteals in the squirrel. The relation of the nerves to the muscle fibres cannot be satisfactorily shown in a microphotograph on account of the thickness of the vessels and the refringency of the fat around them.

In the small lacteals, devoid of smooth muscle, no nerves can be made out. This would suggest that the nerve supply of the lacteal in the squirrel is motor, but we would not, at present, go further than suggesting this as a possibility. We have only examined the nerve supply of the squirrel's lacteals in one animal. The methylene blue method is capricious, and a negative result, with regard to the nerve supply to the endothelial portion, unless repeatedly confirmed, must be accepted with some reserve.

The results here recorded on the nerve supply of lacteals are in agreement with the observations of Dogiel (6) and Kytmanof (10) who, however, studied other lymphatics than those of the mesentery.

5. *Discussion.*

From observations recorded elsewhere (7 and 8), it was found that in two species—the rat and the guinea-pig—rhythmically contractile lacteals were present. Other species so far examined under precisely similar conditions have not given any evidence of rhythmicity, though found to be contractile to various stimuli. In searching for a possible cause for this the present histological investigation was made.

It would appear that there is no essential difference detectable in the lacteals of the guinea-pig and rat on the one hand and the cat on the other, except that the two former species have a rather more abundant supply of smooth muscle. When it is said that a lacteal of the guinea-pig is not nearly so rich in muscle as a mesenteric vein of corresponding size, some idea will be gained of the small complement of smooth muscle which is capable of imparting rhythmical propagated contractions to these vessels.

The question arises as to the cause of this rhythmic action. Is it the result of nervous influences, or is the smooth muscle solely responsible ? In preparations from guinea-pig mesentery no nerve cells have been rendered visible by the methods used, but, on the other hand, in the squirrel, definite nerve cells are present. It is therefore difficult to deny the possibility that the rhythmicity present in the guinea-pig's lacteals may owe its origin to the presence of nerve cells—though none have been seen in our preparations.

One species—the squirrel—has a very scanty supply of smooth muscle. It is only in vessels above 400 μ that any can be detected. In the smaller lacteals of the cat also no smooth muscle can be found. These vessels have been seen to contract when stimulated by a strong faradic current or mechanically. It must be confessed that neither of these stimuli are physiological, and that the vessels without muscle nuclei do not contract to such drugs as adrenalin or pituitrin. Nevertheless, it would appear that one is dealing with vessels which are capable of contraction, though possessed only of an endothelial wall. There can be no question of the presence of such cells as the Rouget type to account for contraction. Smooth muscle, after the most careful search has not been found.

Tarchanoff (15), at the same time as he described the contraction of blood capillaries in the tadpole's tail, described similar though slower contractions in the lymphatic capillaries. The contention of Florey and Carleton (9) and E. R. and E. L. Clark (4, 5) that capillary contraction is quite independent of the presence of Rouget cells is strengthened by the above observations.

6. *Summary.*

1. The histological structure of the mesenteric lacteals of the guinea-pig, rat, cat, mouse, hedgehog, dog, pig and squirrel is described.

2. There is no histological evidence to show why the lacteals of the rat and guinea-pig are contractile and those of other species under the same conditions are not.

3. It is pointed out how small an amount of smooth muscle is responsible for the rhythmic contraction in the rat and guinea-pig.

4. The lacteals of the squirrel of less than 400 μ in diameter do not possess any smooth muscle. They are, however, contractile to electrical and mechanical stimuli. These phenomena are adduced as additional evidence that some endothelial structures are contractile.

5. The lacteals of the guinea-pig and squirrel have been shown to be innervated. The nerves are probably motor. Nerve cells have also been found on the vessels in the squirrel.

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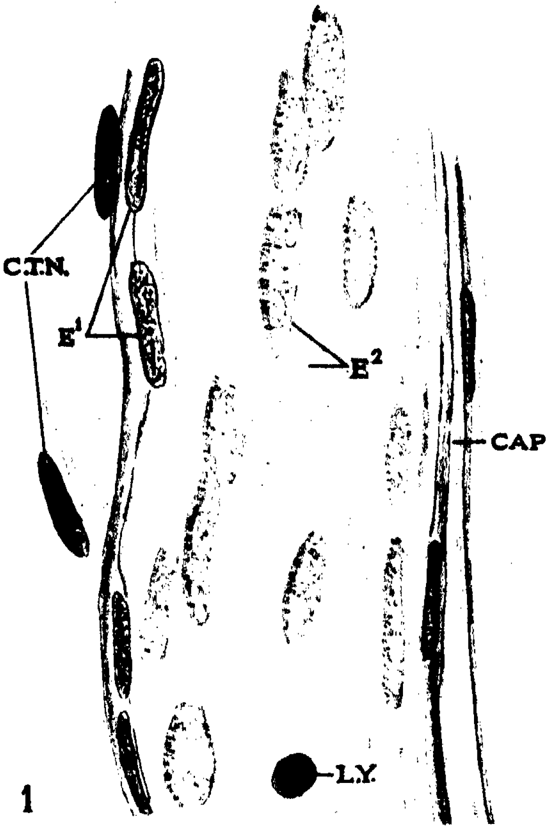

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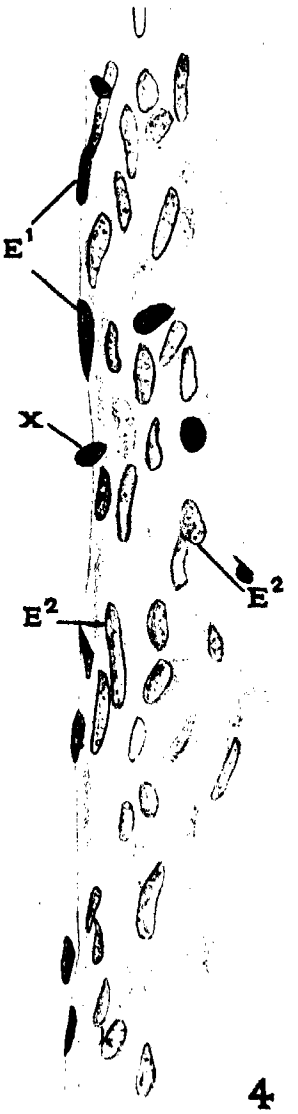

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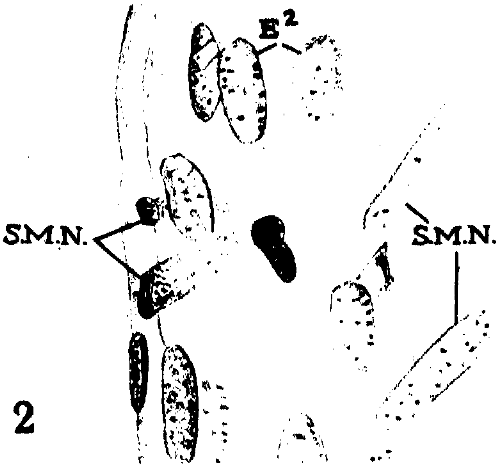

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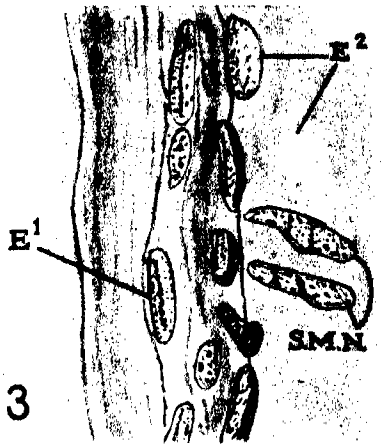

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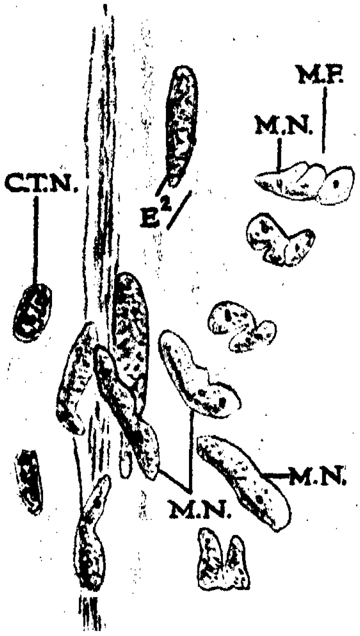

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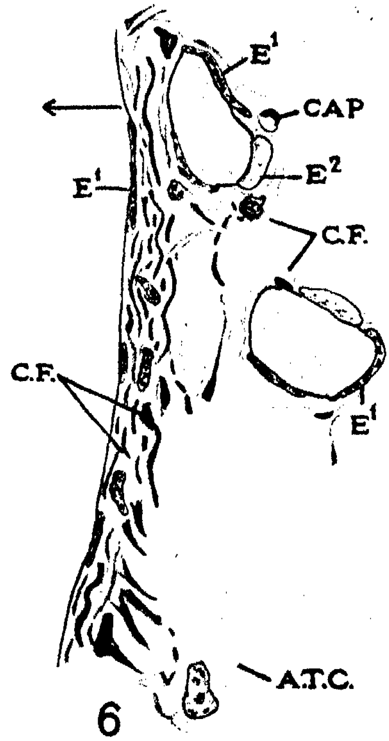

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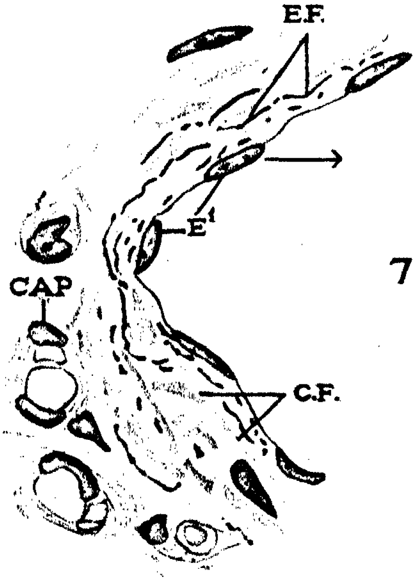

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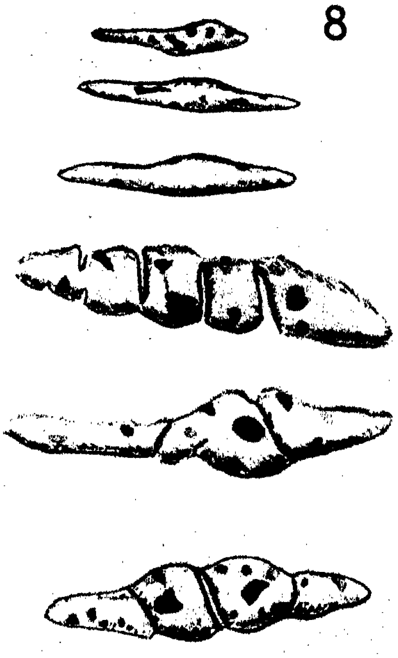

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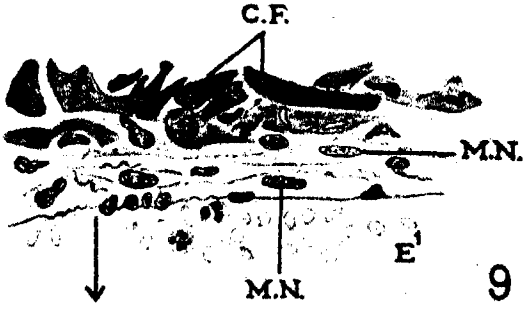

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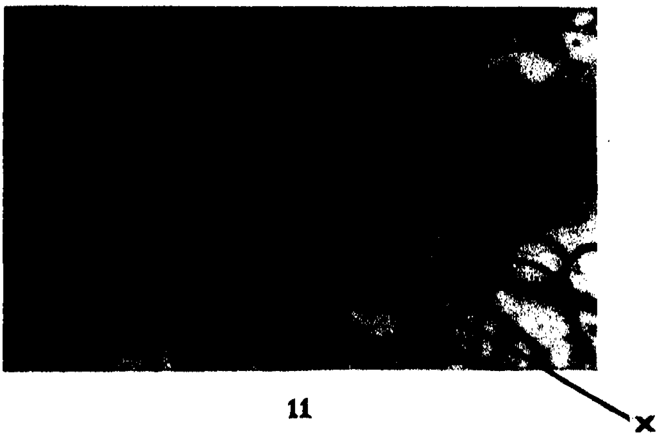

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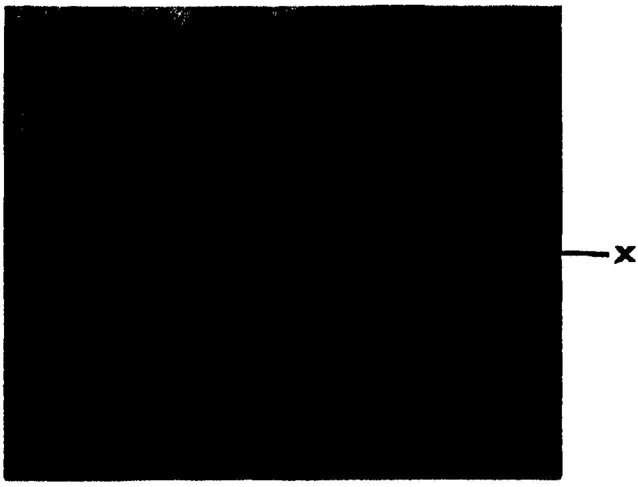

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(8) EXPLANATION OF PLATES.

PLATE 3.

All the figures are drawn with the Abbe camera lucida. Nos. 1 *to* 5 are *from* "whole preparations" of mesentery; in these the uppermost nuclei or cells are depicted darker than the underlying elements. Both endothelial surfaces (*i.e.*, upper and lower with regard to the objective) are conventionally demarcated in this manner, except in fig. 5, in which only the upper wall of the vessel is shown. Figs. 6 to 9 are all from sections of 5 μ in thickness.

The arrows point towards the lumen of the lacteal.

Lettering.—A.T.C. = adipose tissue cell; CAP = capillary blood-vessel; C.F. = collagen fibres; C.T.N. = connective-tissue nucleus; E¹ = endothelial nucleus seen in profile; E² = endothelial nucleus seen "en face"; E.F. = elastic fibres; LY = lymphocyte; M.F. = outline of smooth muscle fibre; S.M.N. = smooth muscle nucleus; ×, see the explanation of the figure.

FIG. 1 (× 1050).—A small lacteal in a whole preparation of mesentery of cat. Transverse diameter circa 30 μ. Formol; Weigert's iron hæmatoxylin—van Gieson.

Endothelial (E¹ and E²) and connective-tissue nuclei (C.T.N.) can be seen, but no smooth muscle nuclei. The length of this vessel is 3 mm.; no smooth muscle nuclei or fibres can be seen anywhere along it.

FIG. 2 (× 1050).—A portion of the wall of a fair-sized lacteal in the same specimen. Diameter circa 125 μ. Smooth muscle nuclei (S.M.N.) are here present, in addition to those of the endothelium and connective tissue of the lacteal. The smooth muscle elements are only slightly, if at all, contracted. Hence the spiral nuclear incisions, so often shown in fixed and stained specimens of smooth muscle (see fig. 8) are not in evidence.

FIG. 3 (× 1050).—Another portion of the wall of a large lacteal of the cat. Diameter circa 220 μ. Formol; Weigert's iron hæmatoxylin. Two characteristic smooth muscle nuclei (S.M.N.) are seen lying outside the endothelium transversely to the long axis of the vessel. Spiral nuclear incisions are shown in these.

FIG. 4 (× 525).—Portion of the wall of a fair-sized lacteal of the squirrel. Whole preparation; formol; Weigert's iron hæmatoxylin. Diameter of vessel circa 110 μ.

Endothelial nuclei (E¹ and E²) can be seen; so can dark nuclei (X), which, on focussing, are seen to form the pavement epithelium of the mesentery. Muscle nuclei or fibres cannot be made out in this or in other lacteals below and up to this size in the squirrel.

PLATE 4.

FIG. 5 (× 1050).—Part of the wall of a whole preparation of the lacteals of the rat. Formol; Weigert's iron hæmatoxylin. Abundant smooth muscle nuclei (S.M.N.) are present; their orientation is transverse or oblique to the long axis of the vessel; some of them show typical spiral nuclear incisions. The outlines of some of the muscle fibres can be faintly seen at M.F.

FIG. 6 (× 525).—Transverse sections of part of the wall of one large and two small lacteals of the cat. Formol; Weigert's iron hæmatoxylin—van Gieson. The endothelial lining (E^1) and the thin wall of delicate collagen fibres (C.F.) are shown in the larger vessel. Nuclei are scattered amongst the collagen fibres; the former are connective tissue rather than muscle nuclei.

The two small lacteals show only endothelium (E^1 and E^2), and a very delicate investment of collagen fibrils (C.F.).

FIG. 7 (× 1050).—Slightly oblique section of the wall of a lacteal in the cat. Formol; Weigert's elastin stain—lithium carmine.

The presence of elastic fibres (E.F.) as well as collagen ones (C.F.) is shown. So also are the capillary blood-vessels (CAP), often noted in close proximity of lacteals.

The arrangement of the elastic and collagen fibres, when studied in longitudinal sections or in whole preparations of the lacteals, is mainly parallel to the long axis of the vessel. But cross anastomoses are very frequent in the elastic meshwork.

FIG. 8 (× 2100).—Illustrates the differences between the nuclei of smooth muscle fibres and those of the connective tissue in the wall of a vein in a section of the cat's mesentery. Formol; Weigert's iron hæmatoxylin—van Gieson.

The vein being contracted, the characteristic spiral nuclear incisions of the smooth muscle nuclei are clearly shown. Three connective-tissue nuclei above, three smooth muscle nuclei beneath.

FIG. 9 (× 525).—Transverse sections of a medium-sized vein in the cat's mesentery. Formol; Weigert's iron hæmatoxylin—van Gieson.

Obvious smooth muscle nuclei (S.M.N.) and fibres can be seen in the tunica media. The collagen fibres (C.F.) of the tunica adventitia are far stouter than in the lacteals.

PLATE 5.

FIG. 10 (× 81).—Lacteal of guinea-pig, 200 μ in diameter. Nerve fibres longitudinal to the main axis of the vessel. The very refringent perilymphatic fat is well shown.

FIG. 11 (×81).—Lacteal of squirrel, 220 μ in diameter. The nerve fibres here are mainly transverse. At × is a nerve cell.

FIG. 12 (× 354).—The same preparation at a higher magnification. Illustrating the fine anastomoses between the fibres. At × is the nucleus of a smooth muscle fibre.

All the above microphotographs were taken by Mr. F. Haynes with a Leitz "Micca" camera. They were subsequently enlarged by Mr. Wm. Chesterman. The present approximate magnifications are given for each figure. The microphotographs are untouched; they all represent nerves in the lacteal wall stained by vital intra-peritoneal injections of methylene blue and fixed by the ammonium picrate method.

Measurement of the Biologically Active Ultra-Violet Rays of Sunlight.

By Leonard Hill, F.R.S.

(Received February 2, 1927,—Revised June 8, 1927.)

(From the National Institute for Medical Research.)

[Plate 6.]

Methods of Measurement.

The ultra-violet radiation was measured by the acetone methylene-blue method described by Webster, Hill and Eidinow.* Readings were taken at the Meteorological Office, Kingsway, The Royal Observatory, Greenwich, the States Meteorological Station, Guernsey, the National Institute for Medical Research, Hampstead, and by voluntary observers elsewhere, viz., by Dr. Adams, Liverpool Sanatorium, Frodsham, Cheshire; Dr. Paul Tozer, Peppard Common Sanatorium, Oxon; Dr. Segaller, St. Ives, Cornwall; A. R. Tankard, Esq., Hull; Dr. Wyatt, Wingrave, Lyme Regis; H. J. Sargent, Esq., Bexhill; C. L. Rivers, Esq., Harrogate; Dr. Dorno, Davos, Switzerland; J. Fyfe, Esq., Stirling; S. M. Bower, Esq., Huddersfield; R. Robertson, Esq., Lowestoft; F. R. Gray, Esq., Ventnor; R. L. Massey, Esq., Khartoum; Dr. P. W. Moore, Assouan; Dr. Rosenheim (some records in Egypt).

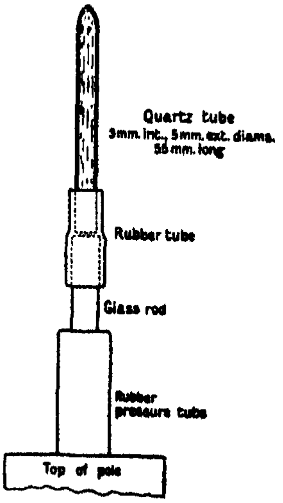

Fig. 1.—Standard Silica Tube and Mounting.

The standard acetone methylene-blue is made up from a 0·1 per cent. solution of Paulene Frères methylene-blue; 5·8 c.cm. of this is added to 30 c.cm. of pure distilled acetone, and made up to 100 c.cm. with water; this gives a standard labelled 10. The standard solution is kept in brown glass bottles. A standard silica tube, as shown in fig. 1, 3 mm. in diameter, is filled with this solution and exposed to the sunlight. At the end of the exposure the colour is compared with the colour of eight standard tubes (fixed and unbleachable). These standard tubes are marked 3, 4, 5, 6, 7, 8, 9 and 10, and are placed in a suitable frame for comparison.* The standard solution used for bleaching in the silica tube is matched against these standard tubes, not only after the period of exposure, but also before exposure, since the standard solution (10) may very gradually fade, while also the refractive power of the silica tubes may vary.

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* *Lancet*, vol. 206, p. 745 (April, 1924).

The difference between the readings, before and after exposure, gives a measurement of the shorter wave-lengths of the ultra-violet sunlight for the time and place of exposure.

A solution of acetone in water is decomposed by ultra-violet light, with the formation of acetic and formic acids, and other substances. The extent of this decomposition can be measured by means of the reduction of methylene-blue, which acts as a hydrogen acceptor in this reaction. Exposed to a constant source of ultra-violet rays, the bleaching of the methylene-blue is proportional to the time of exposure. The acetone-blue solution in a 3 mm. layer transmits λ 3342, partly absorbs λ 3132 of the spectrum of the mercury-vapour lamp, and wholly absorbs rays shorter than λ 3132 (fig. 2, Plate 6).

The degrees of the colour scale are equal and were biologically standardised, using a mercury-vapour lamp, against both the killing of infusoria in a quartz cell and the erythema dosage of the white skin. In the case of the mercury vapour lamp one unit of fading on the scale is equal to twice the infusoria-killing dose, and to 2–4 times the erythema dose, the sensitivity of skin varying in individuals. The skin of the inner surface of the upper arm was used for the erythema dose. How far this quantitative scale can be applied to sunlight is considered later.

Dr. A. B. Hill has tested for us, among 53 people, the accuracy in matching the acetone-blue of different tints against the fixed scale, and found it of quite high order, 80 per cent. of the readings lying within a quarter of a unit of the mean. The temperature coefficient was determined between 1·5 and 35° C., and found to be 1·15 to 1·2 for 10° C. A difference of 10° C. above or below 20° will make a difference of about half a degree of the scale. Such a correction may be required in the tropics or cold climates.

Fig. 1 shows the correct method of mounting the tube. At Hampstead the tube is thus mounted on the end of a long bamboo, hinged at its bottom, and supported by movable guys. This allows the tube to be brought down for changing the solution, and supports it clear of all roofs and obstructions.

Ultra-violet rays reach us diffusely from the blue sky and bright clouds as well as direct from the sun. Dorno has shown that the ultra-violet radiation from the clear sky at Davos is greater than that from the low sun. Taking the diffuse ultra-violet from the sky as 100, that from the direct sun was found by Dorno to be :—

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* The apparatus is made by Siebe, Gorman, Ltd., 187, Westminster Bridge Road, S.E.1.

Height of Sun.	*U.-V. Radiation.*
10°	1·7
20°	12·3
40°	47·6
60°	85·1

The silica tube containing the standard acetone-blue solution must then be exposed to the sky right down to the horizon line. Fig. 3 shows the difference between the readings taken on the roof and in the grounds at Hampstead.

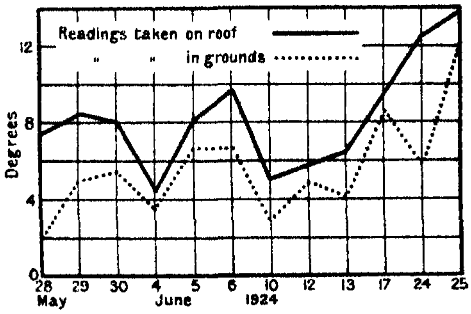

FIG. 3.—Effect of Skyshine.

At one station the reading appeared to be 30–40 per cent. too low. The full exposure of the tube to the sky was found to be obstructed by trees. It was

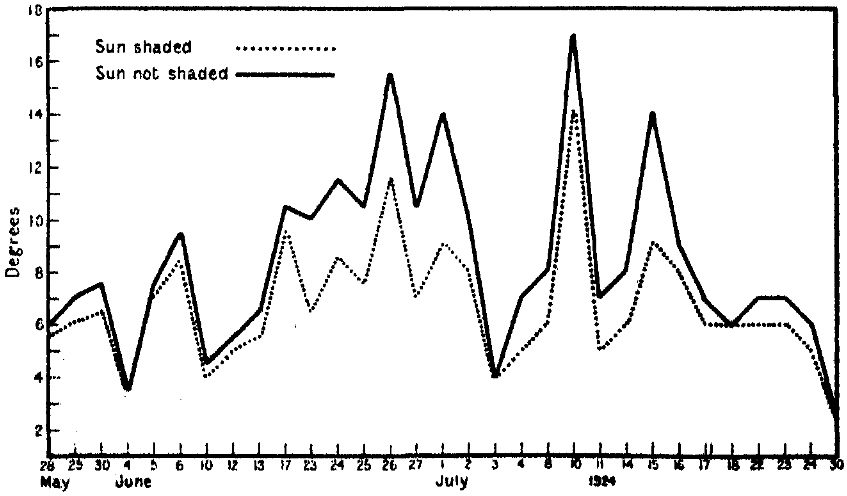

FIG. 4.—Effect of Skyshine. Comparative Readings with moving Horizontal Quartz Tube.

agreed that the tube should be exposed in future on the top of a tall mast. The readings continued to be too low. The tube was then found to be screened from the northern half of the sky by an obstruction on the top of the mast. On raising the tube above the obstruction the true readings were obtained.

Fig. 4 shows records taken by the acetone-blue method with two silica tubes at Hampstead. The tubes were suitably affixed for me by Mr. T. C. Angus to a clock, so as to be carried round in the horizontal position and kept normal to the sun. A small strip of brass attached sufficed just to shade one tube from the direct sun. Thus, one tube was exposed to the skyshine and sun, while the other was shaded from the direct sun, and exposed to the skyshine only. The graph shows that the ultra-violet rays come mostly from the skyshine.

Ultra-Violet Rays of Sunlight which fade Acetone-Blue.

While the spectrum of the sun may extend to λ 2900, λ 3000 is the practical limit. Using a Hilger quartz spectroscope and a bromine screen, we have found the spectrum of the sun scarcely reached λ 3000 at Hampstead. A saturated solution of uric acid (1 in 40,000) in a stratum 6 cm. thick absorbs all rays of shorter wave-length than λ 3200,* cutting out from the spectrum of the mercury-vapour lamp the intense band λ 3132 (fig. 2). Exposing under such a stratum a sealed silica tube, full of the standard solution of acetone-blue, to a mercury-vapour lamp, a similar tube being under distilled water, the fading of the latter was 6, while that of the former was insignificant. On another occasion the fading was 9 under water, 1 under uric acid solution.

On the other hand, exposing similarly the tubes to sunlight, the fading on several days in May at Hampstead on the top of the hill was :—

Under uric-acid solution.	Under distilled water.
3·0	3·5
3·5	4·5
4·5	6·5
4·0	5·0
5·0	6·0
Average.... 4·0	Average.... 5·1

While the active rays for fading acetone-blue of the mercury vapour lamp are then λ 3142 and of shorter wave-length than this, those of the sun in England are chiefly of longer wave-length than λ 3142. (In one observation carried out for us at Assouan by Dr. P. W. Moore, the fading was twice as great under water as under uric acid, an indication of the greater intensity of the shorter wave-lengths of the ultra-violet rays of the sun there.)

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* According to Dhéré this solution absorbs all rays shorter than λ 3060.

Under 3 per cent. quinine sulphate solution, which absorbs rays of the mercury vapour lamp shorter than λ 4200 (fig. 2), the fading of the acetone-blue in sunlight during a certain period was found to be insignificant, while under distilled water it was 4½. The rays of the sun in England, active in fading acetone-blue, are certainly then of shorter wave-length than λ 4200. As the acetone-blue solution partly absorbs λ 3132 and some of the other rays of shorter wave-length than λ 3342 (fig. 2), it must be rays between λ 3342 and λ 3000 which produce the fading in sunlight.

The Erythema-Producing Rays of Sunlight.

Sunlight focussed on the skin through the solution of quinine sulphate does not produce erythema if the skin is kept cool by running water. The erythema-producing rays are then in the ultra-violet region. Further, to determine these rays the effect of Chance's Vitaglass as a screen was compared with that of silica and of green window glass (fig. 5, Plate 6). Vitaglass and green window glass were found by Bourdillon to have the following percentage transparency :—

	Thickness in mm.	Wave-length in Ångstrom units.								
		λ 4046	3655	3342	3131	3022	2967	2893	2804	2655
Green window glass.	2·8	87·0	76·6	26·0	0·12	—	·01	—	—	—
Vitaglass[†] (1926 sample).	1·3	91·0	89·1	86·5	66·5	48·1	34·5	18·6	5·3	0·4

[†] The transmission of recent samples has been improved.

Exposed for two hours to the mid-day summer sun, the skin of the abdomen reacted with a slight erythema under green window glass, distinct under Vitaglass, well marked under silica or no screen (fig. 6, Plate 6).

As silica lets through about 90 per cent. all along the U.V. spectrum, while Vitaglass let through about 75 per cent. of the rays between λ 3300 to λ 3000, and green window glass let through 25 per cent. of those rays at λ 3342 and little below λ 3200,[*] and as in the sun a slight production of erythema occurs under green window glass, a greater one under Vitaglass, and still greater under silica, we may conclude that the erythema-producing rays of the sun mainly lie between λ 3300–3000. Chance's ultra-violet glass transmits rays particularly about λ 3600, and no erythema results under this.

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[*] Coblentz and Fulton, 'Bur. Stds. Sci. Paper, No. 495,' fig. 4 (1924).

Exposed to the summer sun the average fading of acetone-blue on a number of days was 8½ under silica, 7½ under Vitaglass, 4 under green window glass. There is then a correspondence between the acetone-blue fading and the erythema-producing power of the sun's rays, and, in fact, exposure to the summer sun about noon, sufficient to fade the acetone-blue one on the scale, produces well-marked erythema.

While as a quantitative method the fading of acetone-blue in sunlight can be claimed to give only approximate values, it enables one to compare in a qualitative way the value of the sunlight in various places.

In the case of pure acetone, Henri* has shown that the maximal absorption is

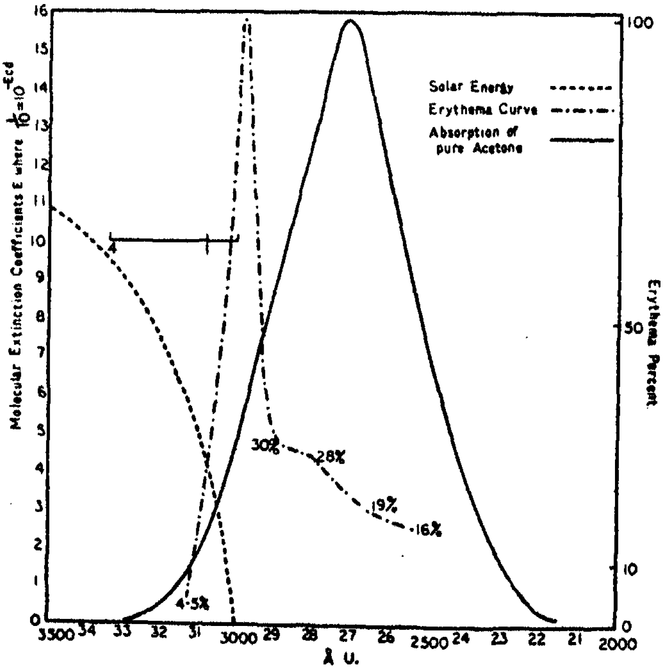

Fig. 7.

at λ 2706 (fig. 7). Hausser and Vahle,† using the bands of the spectrum of the mercury-vapour lamp and equal intensities as determined by the thermopyle, have found the maximal erythema production to be at λ 2967. At λ 3131 the erythema production was only 4·5 per cent. of that at λ 2967. While the erythema-producing action of rays about λ 3131 are then relatively weak, the intensity of the sun in this region is sufficient to be effective. So, too, in the case of the fading of acetone-blue. The rays of the sun which activate ergasterol, and by so forming vitamin D prevent rickets, lie between λ 3200 and λ 2900.

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* 'Compt. Rend.,' vol. 156, p. 230 (1915).

† 'Strahlentherapie,' vol. 13, p. 59 (1922).

In the case of biological reactions produced by ultra-violet rays there is a minimal value below which no biological reaction results, (*a*) for time, (*b*) for intensity of the source. While interrupted excitations, each below the threshold value, may be summated and provoke a reaction, with sufficient intervals such interrupted excitations become ineffectual. Weak ultra-violet radiation which produces a fading effect on acetone-blue in the course of 24 hours may therefore have no erythema-producing effect. It is known, however, that a curative effect can be produced in the absence of erythema.

Records of the Biologically Active Ultra-Violet Rays.

Tables I, II and III show the average monthly readings taken with the acetone-blue gauge for 1925 and 1926 and first five months of 1927 in the places of observation. The highest British ultra-violet reading obtained so far has been 23, at Peppard, on July 10, 1924. At Hampstead the reading on this day was 18. Fig. 8 shows the monthly daily averages for Hampstead, Peppard

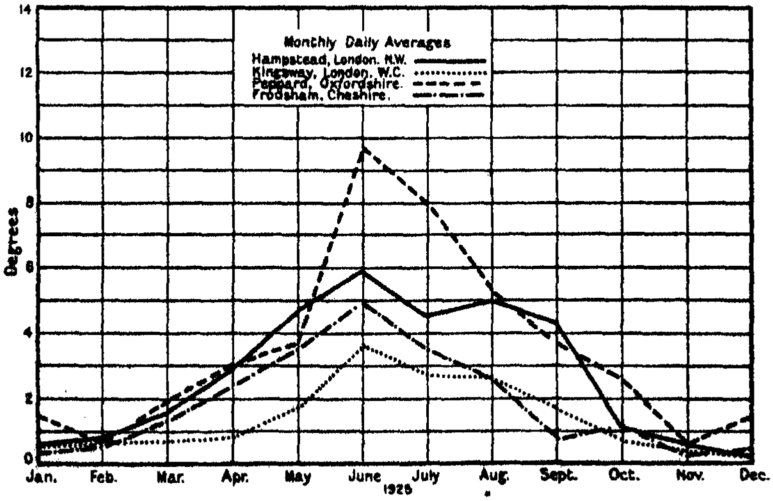


FIG. 8.—Monthly Daily Averages.

(Oxon), Kingsway and Frodsham (Cheshire). The readings are highest in the summer and lowest in the winter months.

Places remote from towns, such as Peppard Common and the top of Hampstead, which is singularly free from smoke during the greater part of the year owing to its height and position to the North-west of London, receive considerably more ultra-violet light than those more exposed to the smoke clouds of industrial areas, *e.g.*, Kingsway and Hull. Ultra-violet intensity is clearly more dependent upon artificial air pollution than upon natural causes, due to geographical position, in England. The readings in Davos and still more in Assouan gain greatly from geographical position. At Assouan the readings in the winter months are very high. The readings at Davos or Assouan on clear days may not be higher than those in summer in clean country or seaside places in South England, but the monthly average is much higher in the first two places, and particularly so in Assouan. On two humid, overcast days at Assouan the readings were 3 and 2, against 12 to 18 for clear sunny days. At Ventnor a reading of 5 was obtained on the sea front, bathed in sun, and 1 on the cliffs, shrouded in mist, on the same day.

A series of readings was taken on the East Coast in August, with one tube at the edge of the cliff exposed to the seashine, and the other inland. As no difference in reading was found, it was concluded that the ultra-violet rays reflected from the sea, at least on the East Coast, are insignificant. Periodic variations of ultra-violet radiation have been recorded by hourly readings at Khartoum during continuous sunshine on some days, with no obvious cause.

Table I.—Ultra-Violet Light. Daily Average for the Month.

Place.	1925.											
	Jan.	Feb.	Mar.	Apl.	May.	June.	July.	Aug.	Sept.	Oct.	Nov.	Dec.
Hampstead	0·6	0·8	1·6	3·0	4·8	5·9	4·5	5·0	4·3	1·1	0·6	0·2
Peppard (Oxon)	1·5	0·6	2·0	3·0	3·7	9·7	8·0	5·3	3·7	2·6	0·6	1·6
Kingsway	0·6	0·6	0·7	0·8	1·7	3·6	2·7	2·6	1·8	0·7	0·4	0·2
Frodsham (Cheshire)	0·4	0·5	1·3	2·4	3·5	5·0	3·5	2·6	0·8	1·1	0·4	0·5
Harrogate	0·6	0·8	1·2	2·1	4·1	5·2	6·1	4·5	—	—	—	—
Hull	0·2	0·3	0·9	1·1	2·8	3·4	3·3	2·5	1·5	0·7	0·4	0·2
St. Ives (Cornwall)	—	1·3	2·1	3·0	3·4	10·5	—	—	—	—	—	—
Greenwich	0·7	1·0	1·5	2·7	3·5	4·0	4·3	4·2	3·3	2·5	1·6	1·1
Lyme Regis	—	—	1·6	3·1	5·8	7·7	—	5·9	5·3	2·9	—	0·8
Guernsey	—	—	—	—	3·7	—	—	—	—	4·2	1·9	1·5
Huddersfield	—	—	—	—	—	—	4·5	3·3	1·9	0·7	0·5	0·2
Stirling	—	—	—	—	—	—	—	—	—	1·9	0·8	0·5
Bexhill	—	1·2	2·0	3·3	5·2	4·6	2·9	3·7	2·7	1·7	1·2	0·7
Davos (Switzerland)	—	—	—	—	—	—	—	—	6·3	—	3·0	1·9

Table II.—Ultra-Violet Light. Daily Average for the Month.

Place.	1926.											
	Jan.	Feb.	Mar.	Apl.	May.	June.	July.	Aug.	Sept.	Oct.	Nov.	Dec.
Hampstead	0·4	0·6	1·8	2·9	4·4	5·3	6·2 (11)	5·8 (3)	3·6	1·3	0·3	0·4
Peppard	0·6	1·0	2·8	3·3	3·2	6·5	6·8	5·0	3·1	2·1	0·8	0·9
Kingsway	0·3	0·3	1·2	2·0	1·7 (25)	3·2	3·4	3·6 (26)	2·5	0·8	0·6	0·2
Frodsham (Cheshire)	0·3	0·4	0·7	3·0	3·3	3·8	3·3	3·2	—	—	—	—
Hull	0·3	0·4	0·4	1·2	2·0	2·5	3·7	3·6	2·2	1·7	0·4	0·3
Greenwich	1·0	—	—	—	—	—	—	—	—	—	—	—
Lyme Regis	0·7	1·1	2·4	3·8	5·0	6·9	7·2	6·9	5·2	2·3	1·5	1·5
Guernsey	1·8	2·2	2·0	2·4	4·1	6·9	6·4	4·8	3·7	2·3	1·4	0·7
Huddersfield	0·2	0·5	0·9	1·2	2·1	3·2	3·8	3·1	2·2	1·3	0·6	0·4
Bexhill	0·7	1·2	2·2	2·7	3·1	4·8	6·9	6·3	4·8	2·0	1·6	2·0
Ventnor	—	—	—	—	(12)	—	—	6·7	5·9	3·4	2·5	2·5
Lowestoft	0·6	0·4	1·5	1·8	5·0	4·8	6·7	6·6	5·9	4·8	1·2	1·3
Stirling	0·2	0·5	0·9	1·3	3·0	4·2	6·0	5·4	4·1	2·1	0·8	0·3
Davos (Switzerland)	1·7	2·0	2·8	5·1	5·9	6·0	9·0	10·4	8·4	4·4	3·5	2·3
Agra	—	1·0	2·0	2·5	3·8	6·0	9·5	7·7 (8)	6·8 (16)	2·5	1·5	1·3
Egypt (Khartoum)	—	—	—	—	—	—	—	13·0	10·6	—	—	—
Assouan	—	—	—	—	—	—	—	—	—	—	—	11·4

The figures in brackets indicate number of observations in the month. At Lowestoft up to August the readings were too low, owing to screening of skyshine by trees.

Table III.—Ultra-Violet Light. Daily Averages for the Month.

Place.	1927.					
	Jan.	Feb.	March.	April.	May.	June.
Hampstead	0·39	0·55	1·34	2·55	3·9	3·22
Peppard	0·97	0·97	2·10	3·56	5·87	—
Kingsway	0·21	0·21	0·74	1·50	3·45	2·57
Hull	0·18	0·23	0·55	0·86	1·43	2·13
Huddersfield	0·48	0·89	1·97	2·83	2·69	—
Bexhill	1·55	1·39	2·74	4·13	5·27	3·88
Lyme Regis	1·18	1·71	3·52	4·50	4·71	4·72
Lowestoft	1·50	1·39	3·16	2·96	3·48	3·66
Guernsey	0·97	1·82	2·61	3·36	4·96	6·33
Ventnor (I.O.W.)	2·77	2·48	3·29	3·86	5·19	5·42
Torquay	—	—	—	3·45	4·05	4·50
Dagenham (Essex)	—	—	—	2·36	5·43	4·75
Davos (Switzerland)	3·00	4·25	4·55	7·60	7·72	5·92
Assouan (Egypt)	12·90	12·75	—	—	—	—

Summary.

Daily values of biologically active ultra-violet radiation, taken during 1925, 1926 and part of 1927 at many places, show that there is a great loss of this radiation in smoky cities. The winter readings are much lower than the summer.

While Davos in Switzerland receives a greater amount of ultra-violet radiation than the most favoured British place, Assouan in Egypt receives a greater amount than Davos.

I am indebted to Mr. C. Pergande for his care in keeping the records.

On Intracellular Oxidation-Reduction Potential.

By Louis Rapkine and René Wurmser.

(Communicated by Sir Frederick Hopkins, F.R.S.—Received July 12, 1927.)

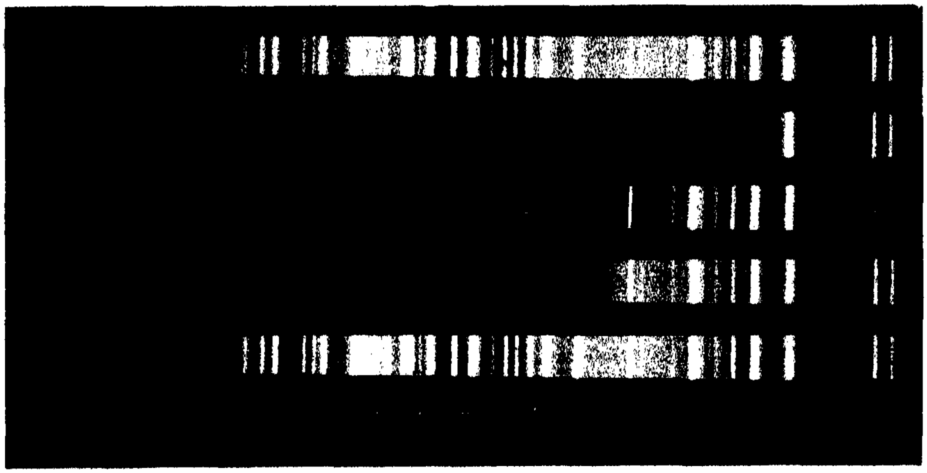
(From the Laboratory of Biophysics, Collège de France, Paris.)

Introduction.

Two types of oxidations are to be distinguished in living cells; oxidations at the expense of free oxygen, and oxidations at the expense of oxygen derived from complex molecules. The former are what we have named "*independent*" in contrast to the latter, which are named "*coupled*" reactions (1). The independent oxidations may also be called respiratory in nature; they are not utilised as sources of energy except in the special case of homoiothermic animals which are below the point of thermic neutrality. The coupled oxidations are accompanied by reductions which constitute the real syntheses occurring in cells. Only these oxidations are able to compensate for the chemical work represented by the syntheses, as well as the mechanical work derived therefrom.

The power of cells to carry out syntheses is thus dependent on the property of certain substances to be oxidised at the expense of oxygen borrowed from complex molecules. In other words, if we admit with Wieland (2) that oxidations begin by a mobilisation of hydrogen, the possibility that syntheses may take place depends upon whether the hydrogen is capable of being fixed by *an acceptor* other than molecular oxygen.

To take a concrete example, we must look for the conditions which permit the hydrogen liberated by a sugar to be fixed by a system consisting of ketonic acid + ammonia rather than by a system consisting of free (molecular) oxygen.



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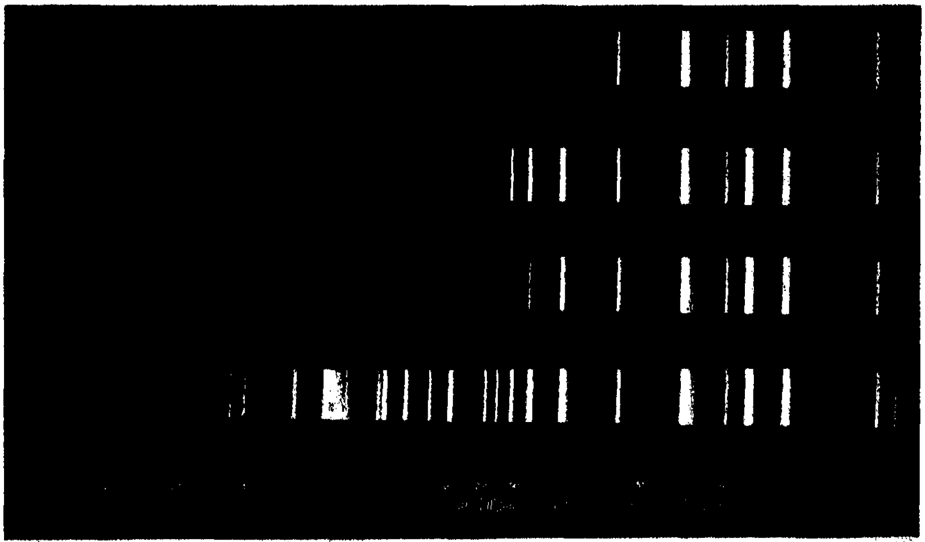

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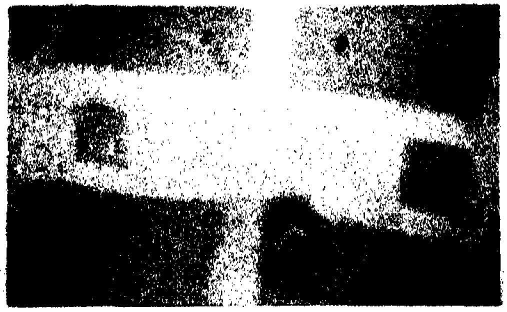

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1. Vitaglass.
2. Chance's ultra-violet glass.
3. Green window glass.
4. Silica.

In the first instance we have a synthesis of alanine, while in the second there will only be a production of CO_2 and H_2O. It has appeared to us that there may exist a relation between the power of organisms to bring about these oxido-reductions and the oxidation potential of the intracellular substance.

Before going farther, we wish to insist that, in general, there is no relation between the speed and consequently the effective realisation of reactions and their potentials. However, if conditions of catalysis are assumed to be identical, it is not illegitimate to look for such relations. We have learned from Wieland to consider the essential act of an oxidation to consist of a mobilisation of hydrogen. This hydrogen can, at least in the well-studied case of activation by xanthine oxidase (Dixon (3)) be fixed by any acceptor. It suffices that this acceptor have a structure more or less labile, and that, for example, the system ketonic acid + ammonia be more labile towards the hydrogen than is the molecular oxygen. Whence the possibility of an oxido-reduction. But there exists within the cell a relatively large quantity of acceptors capable of entering into reversible combination with hydrogen; and if the cellular substance is in some sort of way *buffered*, in this respect, as it is in respect to hydrogen-ion concentration, the system ketonic acid + ammonia will be reduced only if its reduction potential is superior to the potential of the cell protoplasm.

If we consider the synthesis of alanine, which we have already taken as an example, we see that the increase of free energy that accompanies such a synthesis, according to the equation

$$2CH_3CO\,.\,COOH + 2NH_3 \longrightarrow 2CH_3CHNH_2COOH + O_2 \text{ (1 atmosphere)}$$

(supposing that Berthelot's principle may be applied), is about 60,000 calories. If this synthesis is compensated by an oxidation of the hydrogen, it may be calculated* that the pressure of this hydrogen must be at least 10^{-20} atmospheres in order that the variation of free energy accompanying this oxidation be 60,000 calories. Now, a hydrogen pressure of 10^{-20} atmospheres

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* The variation of free energy corresponding to the reaction

$$2H_2 + O_2 \text{ (1 atmos.)} \longrightarrow 2H_2O \text{ (0,019 atmos.)}$$

is for 2 molecules of hydrogen

$$-\Delta F = RT\left(\log K - \log \frac{pH_2O^2}{pH_2^2\,.\,pO_2^2}\right)$$

where the equilibrium constant K, at 18° C., is equal to $10^{83.27}$. If for $pH_2O = 0{,}019$ atmos. and $pO_2 = 1$ atmos., one plots the different values of ΔF as functions of $rH = \log\frac{1}{pH_2}$, one obtains a curve which is a straight line, on the ordinates of which 60,000 calories corresponds to an $rH = 20$.

corresponds to an $rH = 20$, and this is what Needham and Needham (4) first, and then ourselves (5), have found experimentally in cellular material.

In order that the determination of such a potential have any significance, it is essential that there exist within the cells substances in equilibrium with this hydrogen and that the reactions that ensure such an equilibrium take place at a great velocity. This is what seems to happen, as seen from the results of micro-injection of Clark's indicators into cells, since the same results are attained whether the indicator be injected in its oxidised form or in its reduced form. This is what Clark (6) means when he says that cells are poised.

It is, however, unnecessary that the donator of hydrogen be in real equilibrium with the hydrogen that it liberates. The potential may be but an *apparent* potential, such as that which can be determined in solutions of reducing sugars. These sugars continually liberate hydrogen, which is capable of charging a platinum electrode. The potential thus produced makes it possible to predict which indicators will be reduced by the sugars and which will not be (7). But if we wish such a solution to be buffered or "poised," it is necessary to add a substance, for example, phenosafranine, whose *normal* oxidation-reduction potential is very near the potential of the solution. A model of the cell is thus constructed with regard to oxidation-reduction equilibria.

In a system such as this the hydrogen liberated by the donator can fix itself on to any acceptor only if the latter has a reduction potential superior to that of the substances in rapid equilibrium with the hydrogen, that is, if the acceptor has a potential superior to that of the cell.

It is clear that in a cellular system having an $rH < 20$, reductions corresponding to an elevation of free energy greater than 60,000 calories will be able to take place. One can thus forsee a relation between rH and the nature of chemical reactions that take place within a given cell or cell-phase, that is, the nature of the metabolism.

All that precedes implies that the hydrogen pressure concerned in these reactions is capable of being measured by means of a platinum electrode, that is, the reactions take place by the intermediary of hydrogen ions and electric charges which are in equilibrium with the gaseous hydrogen (M. Clark), or by atomic hydrogen (Dixon (8)).

Of course, only rigorous experimentation will show the validity of such hypotheses. We can, however, adduce in support of the view that there is a relation between rH and the nature of the chemical processes in the cell—the experiments of Knoop (9) on the one hand, and Quastel and Stephenson (10) on the other.

Knoop (9) has shown that the quantitative formation of amino-acids at the expense of keto-acids + ammonia is made possible *in vitro* if the medium is reducing.

Quastel and Stephenson (10), in their paper on 'The Relationship of *B. Sporogenes* to Oxygen,' have shown the importance of the presence of a substance containing the — SH group, or any other reducing substance, such as glucose, in a medium suitable for the proliferation of *B. sporogenes*. The point that we want to bring out, namely, the relationship that may exist between *r*H and the nature of the chemical processes in the living cell, will be best illustrated by the conclusions which Quastel and Stephenson arrive at and state in the following words: "Let us assume that before proliferation of *B. sporogenes*, or of any other cell which leads a *strictly* anaerobic career, can occur, the cell must have attained a certain limiting reduction potential. At any potential more oxidising than this, the cell fails to proliferate. At, or below, this potential, proliferation is possible. The potential may be secured by the presence of a small quantity of an — SH compound, or it may obtain in the presence of glucose or a variety of substances characterised by their reducing behaviour in presence of the cell. The effect of oxygen in preventing the proliferation of strict anaerobes would then be due to its removal of substances responsible for the maintenance of the critical reduction potential with the consequent raising of the potential above the limiting point."

The oxidation-reduction potential seems then to be of great importance, and it is necessary to see how it is maintained within the cell. This leads us to investigate the conditions under which there is, on the one hand, liberation of hydrogen by the donators; and the elimination of this hydrogen, either by diffusion or combination, on the other. We thus find ourselves facing the problem from the same standpoint as F. G. Hopkins (11), that is, we are here dealing with a problem of the mechanism of catalysis.

As we have already seen, the intra-cellular potential is an apparent one in the sense of Dixon (8), and is determined by the relation that exists between the liberation of hydrogen and its disappearance. In this case the disappearance consists of a fixation on an acceptor, whether it be free oxygen or some complex molecule. The oxidation by means of molecular oxygen can, as a matter of fact, be brought about by the intermediary of such a carrier as glutathione, or some other hydrogen-transporter.

Quantitatively, except during periods of synthesis, the oxidations that take place are respiratory and are carried out at the expense of molecular oxygen.

In fact, it is to be seen that the rate of disappearance of the hydrogen is regulated by the rate of activation of oxygen. The necessity of an activation of the oxygen seems to result from the measurements of potential in plant cells.

M. M. Brooks (12) has shown, and we have ourselves confirmed this observation by different methods, that the sap of these plant cells is capable of reducing such dyes as 1-naphthol, 2-sulphonic acid, indophenol. Yet within these cells there circulates oxygen; whether this oxygen is free or in a form only unstable at the periphery of the cell, one can affirm that this oxygen does not combine immediately with the hydrogen furnished by the donators, as does the dye which is in equilibrium with the hydrogen. In these plant cells, in the blastocœle fluid of the sea-urchin (13), and probably in the blood, the oxygen has to be activated, and is activated slowly in comparison with the rate of liberation of the hydrogen.

We have asked ourselves, in the course of our work, whether the nucleus was not a place in the cell where the rate of the activation of the oxygen was particularly accelerated; if this were true, one would expect to find a difference of *r*H, unless the rate of dehydrogenation was accelerated in the same proportion.

In addition to that, we wanted to see if an introduction into the cell of certain substances, which, although not necessarily normally taking part in the intracellular oxidation-reduction equilibrium, are very probably hydrogen donators, could modify appreciably the *r*H by lowering it.

Technical and Experimental Results.

The technique employed was in its general aspects the same as that of J. and D. Needham, that is, micro-injection of Mansfield Clark's oxidation-reduction indicators.* A detailed description of it may be found in their papers on oxidation-reduction potentials.

For the sake of convenience each indicator is allotted a number, and it is by this number that it will be referred to.

No. 1—Potassium indigotine disulphonate.
No. 2—Potassium indigotine trisulphonate.
No. 3—Potassium indigotine tetrasulphonate.
No. 3A—Methylene blue.
No. 4—1-naphthol, 2- sulphonic acid, 2- 6- dichloro indophenol.
No. 5—1-naphthol, 2- sulphonic acid indophenol.
No. 6—0-cresol, 2- 6- dichloroindophenol.
No. 7—0-cresol indophenol.
No. 8—2- 6- dibromophenol indophenol.
No. 9—0-chlorophenol indophenol.

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* We wish to express here our thanks to Dr. and Mrs. Needham, who were kind enough to supply us indicators Nos. 1–8, and to Prof. W. M. Clark, who sent us indicators Nos. 9 and 10.

Before going on to our experimental results, it is perhaps suitable here to discuss some objections brought by M. Dixon against the determination of *r*H in biological systems, and by F. Vlès and his collaborators with regard to *p*H measurements in living cells.

Dixon in his interesting paper on 'Xanthine Oxidase,' VII, points out, with reason, that the velocity of reduction of dyes used as indicators should be taken into consideration. He shows that the dyes containing no sulphonic group are reduced quickly and in about the same time. Indicators 4 and 5, which contain one sulphonic group, are reduced definitely more slowly, whilst dyes Nos. 1, 2, and 3 are reduced very slowly indeed and not in the order that one would expect from reduction potential data. In fact, the order is exactly the contrary, and would seem to show that the effect is due rather to the number of sulphonic groups contained in the molecule, No. 3, which contains four groups, being reduced slowest. Moreover, Dixon points out that most of the measurements by micro-injection (where observation is limited to a short period of time) show an apparent *r*H lying just at the junction of the sulphonated and the non-sulphonated indicators of Clark's series. " A consideration of these facts," says Dixon, " suggests that the apparent results obtained may depend not so much upon the *r*H of the cell as upon whether the indicator used is sulphonated or non-sulphonated."

This we can best answer by giving a concrete example. Indicator No. 5 (sulphonated) remains, it is true, oxidised in most of the animal cells previously examined, whilst Nos. 6 to 9 (non-sulphonated) are reduced. In the plant cell (Valonia, Spirogyra), however, we have a case where the internal *r*H is lower than in the animal cell. In these cells No. 5, when introduced, is *immediately* reduced, that is, with the same velocity as dyes Nos. 6 to 9. In another paragraph Dixon states, " Now a dye which is only slowly reduced in a cell will be reoxidised as fast as it is reduced, by oxygen if this is present, or possibly by other hydrogen acceptors in the cell." Now it is practically an established fact that oxygen circulates in the plant cell, yet it has no effect whatsoever on the maintenance of No. 5 in its oxidised form, in spite of the fact that this dye is sulphonated and is more slowly reduced than dyes Nos. 6 to 9 in Dixon's experiments on xanthine oxidase. We will add that when No. 5 is introduced in its reduced form, it stays reduced.

Vlès (14) and his collaborators' chief objection is the concentration of the dyes used in *p*H measurements. According to Vlès, the reason why J. and D. Needham and ourselves obtain higher figures than he does for the internal *p*H is because we use the indicators in greater concentration. Against this objection we can point to the following experiments :—

(1) We inject into the cell a 1 per cent. solution of phenol-red in its acid form. The colour-change from yellow to orange indicates a *p*H value of about 7·2. Simultaneously, we measure electrometrically, in a quinhydrone electrode, the *p*H of the indicator solution ; it shows an acid reaction. We add NaOH, little by little, making it more alkaline, and always measuring electrometrically. At the same time micro-injections are carried out ; the colour change is always the same. Unfortunately, the electrometric measurements in a quinhydrone electrode cannot be depended upon beyond *p*H 7, but as there is a very slight difference of tint between the dye in the pipette and the dye in the cell (it being slightly more acid in the pipette), we conclude that the *p*H value of the cell is about 7·2. The same experiment is performed with brom-cresol purple. We believe to have thus an electrometric control for our colorimetric measurements.

(2) Besides using micro test-tubes for comparison, we have also adopted the following technique :—

> We place, beside the cell to be injected, drops of buffer solutions ranging from *p*H 6·8 to *p*H 8, electrometrically measured, and approximately of the same diameter as the cell. We inject phenol-red into the cell, and into these droplets of buffer solution. The tint in the cell is identical with the tint in the droplet of buffer solution of *p*H 7·2. The same experiments are performed with cresol-red and brom-cresol purple.
>
> We believe that by the above methods of control we have eliminated Vlès' objections. We are, of course, aware of the fact that there are other factors, such as protein error, etc., to be considered : for these, however, we have not, as yet, any experimental control.

(3) One other thing is worthy of notice. P. Reiss (15) has drawn attention to the fact that certain *p*H indicators are decolorised in systems of given reduction potentials. We have confirmed his results, and measured the potential at which such dyes as phenol red and brom-cresol purple are decolorised. As concerns phenol red, not only is it decolorised at too low a potential ($E_0 = -0 \cdot 515$ at *p*H 7·2) to affect *p*H measurements, but in our case it was not reduced, and remained orange even when injected orange.

From Reiss's measurements of potentials of brom-cresol purple it is reduced at *r*H = 18, at *p*H 5·4, which would, of course, render this dye inadequate for use as a *p*H indicator. However, we have found that it, also, is reduced at *p*H 7·2 at a much lower potential ($E = -0 \cdot 426$), too low to have any effect on our *p*H determinations in the plant cells.

We will now pass on to the experimental results, which we will classify in the following table:—

Animal cells (cytoplasm and nucleus).

Material injected.	*p*H indicators.	*r*H indicators.	*p*H.	*r*H.
Cells of salivary gland of Chironomus larvæ	Cresol-red → yellow Phenol-red → orange Brom-cresol purple → purple	No. 5 → oxid. No. 8 → reduced No. 1 → oxid. No. 2 → oxid. No. 3A → oxid. No. 10 → reduced	7·2 ± 0·2	19–20
Oocyte of sea-urchin (*Paracentrotus lividus* Lk.)	Same dyes and same colour changes	Same dyes and same colour changes	7·2 ± 0·2	19–20
Oocyte of star-fish (*Asterias rubens*)	Same dyes and same colour changes	Same dyes and same colour changes	7·2 ± 0·2	19–20
Cells of salivary gland of larva of *Calliphora erythrocephala*	Same dyes and same colour changes	Same dyes and same colour changes	7·2	19–20
Plant cell.				
Spirogyra	Cresol-red → yellow Phenol-red → yellow Methyl-red → yellow Brom-cresol purple → greenish yellow	No. 3A → oxid. No. 3A → oxid. → oxid. No. 1 } No. 2 } → oxid. No. 3 } No. 5 } → oxid. No. 8 }	6 ± 0·2	14–16

In order to see whether certain substances could, when injected, appreciably modify the intracellular *r*H, we have injected into the cells of the salivary gland of the Chironomus larva the following substances:—

(*a*) Glucose, in concentrations varying from ..	1–6	per cent.
(*b*) Sodium succinate	1–10	,,
(*c*) Sodium pyruvate	1–10	,,
(*d*) Fructose	1–10	,,

The *r*H remains constant at 19–20.

Conclusion.

(1) Our determination of the reduction potential of the plant cell confirms the results obtained by M. M. Brooks on another material and by another method,

and seeing that oxygen is present,* we can conclude therefrom that the oxygen must be activated in order to enter into equilibrium with the hydrogen, as we have pointed out above. We therefore believe that at least in this case, and probably in the blood, the oxidation at the expense of molecular oxygen needs not only a mobilisation of hydrogen in the sense of Wieland, but also an activation of oxygen in the sense of Warburg (16). This is in conformity with the views of F. G. Hopkins (11), in that a mobilisation of hydrogen is not always the sole factor, but that an activation of molecular oxygen may accompany it in order to secure an oxidation.

(2) The comparative determinations by micro-injection of *p*H and *r*H in the nucleus and cytoplasm of various cells have revealed no differences. We therefore believe that either the rate of activation of oxygen is not specially accelerated in the nucleus or that the mobilisation of hydrogen is accelerated to the same extent. In the latter case the fact that the *r*H remains the same does not exclude the possibility that the quantity of substances oxidised may be greater within the nucleus than in the cytoplasm, a view held by many authors (Lillie Unna, Warburg, etc.). In fact, it is quite possible that if hydrogen is activated on some active surfaces, these surfaces are more quickly liberated when a large quantity of active oxygen is present.

(3) The micro-injection of glucose, fructose, sodium pyruvate and sodium succinate produces no immediate modification of the intracellular *r*H. This would seem to show that in the cell the rate of dehydrogenation is, within wide limits, independent of the concentration of hydrogen donators.

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Bacterial Proteins.—The Presence of Alcohol-Soluble Proteins in Bacteria.

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(Communicated by Sir Frederick Hopkins, F.R.S.—Received July 12, 1927.)

In the course of an investigation of the Gram-staining material in Streptococci and Staphylococci, substances giving the general reactions of the alcohol-soluble proteins or gliadins have been isolated. Alcohol-soluble proteins, or prolamins (Osborne (1)), are especially characteristic of the seeds of cereals; among them gliadin of wheat and rye, hordein of barley, and zein of maize are typical examples. The present communication deals with the alcohol-soluble fraction of bacterial protein: the other soluble protein substances will form the subject of a later communication. The work was commenced in 1917 as part of the author's study of war wounds, but had to be abandoned, and was not recommenced until the winter of 1923.

Most investigations on bacterial proteins have been conducted by extracting the bacterial cells with alkaline solutions, with or without heating; such a procedure is likely to modify certain of the proteins, and, as will be seen later, would entirely remove the alcohol-soluble fraction. Bacteria are especially resistant to the solvent action of the majority of biochemical solvents used for the extraction of proteins. The Gram-positive organisms, with the exception of Pneumococci, which are soluble in bile (Heidelberger and Avery (2) used this method in examining the protein of Pneumococci) are especially refractory. Douglas and Fleming (3) extracted Staphylococci with hot acetone and removed the Gram-reacting substance; Dreyer (4) used acetone and boiling formalin. All these processes are likely to alter the residual proteins. Liquid cultures are also unsatisfactory, are bulky to manipulate, and introduce disturbing factors.

A general consideration of the problem suggests that (1) bacterial protein is possibly more resistant to the action of acids than alkalies (many bacteria produce acid and can survive considerable concentration of acid); (2) that the molecule of the Gram-reacting substance is likely to contain polar groups, and that the substitution of these (*e.g.*, the benzoylation of the hydroxy group) might alter the solubility and other properties of the substance.

It was determined to attempt benzoylation in an acid medium, and after many unsuccessful trials a mixture of benzoyl chloride in ether was found to remove the Gram-reacting material from all Gram-positive organisms tested, the reaction proceeding slowly at the laboratory temperature, more quickly at 37° C. After removal of the Gram-reacting material the bacteria are about one-third smaller in size, and are easily soluble in 0·2 per cent. of KOH and other dilute alkalies, and partially in normal saline solution, the filtered solutions giving many of the usual protein reactions. Gram-negative bacilli (*B. typhoid*, *B. coli*, *B. paracoloides* and *M. catarrhalis*) are also rendered soluble in dilute alkalies and partially in normal saline solution by the action of benzoyl chloride in ether.

The general outline of the method used is as follows:—Mass cultures of the various organisms are grown upon agar plates 10 inches by 10 inches, removed from the agar surface, avoiding culture medium, suspended in normal saline, and extracted with benzoyl chloride ether mixture for one to three days. After removal of the benzoic acid formed during the extraction, the bacterial mass, washed with acetone and dried, is ground up and dried. The powder is then extracted with normal saline at 37° C. for 24 hours, filtered, washed with ammonia-free water (saline fraction), suspended in 70 per cent. alcohol for 24 hours at 37° C., and filtered (alcohol fraction). The residue (residual protein), which varies in amount for different organisms, is freely soluble in dilute alkali (0·2 KOH), and gives the usual protein reactions.

The various yields of crude bacterial protein obtained after extraction with benzoyl chloride ether, washing in acetone, and drying, are shown from the following examples:—

Staphylococcus aureus ..	48 10-inch plates	dried powder ..	2·6324
Bacillus coli communis ..	,,	,, ..	2·0900
Bacillus typhosus	,,	,, ..	3·2422

Saline Fraction.—The filtered clear solution gives the biuret, Millon, xanthoproteic, and glyoxylic reactions, and is precipitated by phosphotungstic acid. The sulphur reaction is strongly positive. The solution may give a slight Molisch reaction, but this depends upon the amount of washing; the greater quantity of the Molisch-reacting substance is removed in the preliminary extraction. Only a small amount of precipitate is given with half saturation with ammonium sulphate: full saturation produces a larger precipitate of albumose; the filtrate still retains a small amount of biuret-reacting substance of peptone-like reactions. The total amount of the saline fraction is comparatively small in relation to the alcohol- and alkali-soluble fractions.

Alcohol Fraction.—On evaporating the opalescent alcohol filtrate, the material obtained is a clear, yellowish, brittle mass, resembling clear glue, which grinds up to a grey powder. It is insoluble in dilute acids, absolute alcohol, saline, and distilled water, but is freely soluble in 70 to 80 per cent. alcohol, and very soluble in dilute alkalies. It gives no Molisch reaction when purified. The Molisch-reacting substance is removed in the earlier steps of the process, and evidently belongs to the specific soluble substances described by Heidelberger and Avery (5), Zinsser and Parker (6), Laidlaw and Dudley (7), and will be dealt with in a later communication. The alcohol-soluble substance gives Millon's, xanthoproteic and glyoxylic reactions; it is precipitated by trichloracetic acid, phosphotungstic acid, mercuric iodide, picric acid, tannic acid, and salts of the heavy metals; least by lead acetate. Boiled with strong alkali in the presence of lead acetate, a trace of coloration is produced, which varies with different preparations. On igniting and dissolving the melt, only a weak phosphorous reaction is obtained.

The alcohol-soluble protein was further examined for the possibility of partial benzoylation during the preliminary treatment.

Preparation	217 *B. typhosus*	0·116 grm.
„	218 *B. coli*	0·261 „
„	219 *S. aureus*	0·135 „

were hydrolised separately with 20 per cent. HCl, 20 to 30 c.c., in a Durex flask, with a reflux all-glass hanging condenser.

In 24 hours the hydrolysate was biuret-free; when cold it was transferred to a separatory funnel and extracted with ether for 24 hours with occasional shaking. The ether was removed, evaporated over a water-bath and electric hot-plate; a very small amount of brownish residue remained. The residue was extracted with boiling distilled water and filtered while hot. On subsequent cooling, the fluid remained quite clear. The alcohol-soluble protein does not appear to be benzoylated.

The amount of substance extracted from the original bacterial powder is proportionately large. At present no exact criteria of the content of individual bacteria can be given, but the following give an approximation of the alcohol-soluble and other fraction in the dried organisms.

1. *Prep.* 216. *S. aureus.*

*Weight of extracted, acetone-washed, and dried bacterial powder	0·8820 grms.
Weight of alcohol-soluble fraction estimated by evaporation of 100 c.c. of alcoholic solution over water-bath and drying over H_2SO_4	0·1250 ,,

2. *Prep.* 217. *B. typhosus.*

Weight of extracted, acetone-washed and dried bacterial powder	3·4222 ,,
Alcohol-soluble fraction, evaporation	0·2427 ,,
Alcohol-soluble fraction, estimated from nitrogen content	0·2802 ,,
Alkali-soluble protein after alcohol extraction, dried at 37°	1·2924 ,,

3. *Prep.* 218. *B. coli.*

Weight of extracted, acetone-washed and dried bacterial powder	2·0900 ,,
Weight of alcohol-soluble fraction	0·7603 ,,
Weight of alkali-soluble fraction	0·4840 ,,

4. *Prep.* 219. *S. aureus.*

Weight of extracted, acetone-washed and dried bacterial powder	2·6324 ,,
Weight of alcohol-soluble fraction by drying	0·2079 ,,
Weight of alcohol-soluble fraction by nitrogen estimation	0·2910 ,,
Weight of alkali-soluble fraction	0·7834 ,,

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* These quantities refer to the crude bacterial powder, after the preliminary benzoylation and removal of benzoic acid, and before saline extraction.

The nitrogen was estimated in three samples :—

(i) *B. coli.* *Prep.* 135.
Dried alcohol-soluble protein .. .. .. 0·1476 grm.
= 11·80 N/10 H_2SO_4 = 11·2 per cent. N.

(ii) *B. typhoid.* *Prep.* 193.
Dried alcohol-soluble protein .. .. .. 0·210 „
= 1·7 c.c. N/10 H_2SO_4 = 10·9 per cent. N.

(iii) *S. aureus.* *Prep.* 222.
Dried alcohol-soluble protein .. .. .. 0·1366 „
= 11·2 N/10 H_2SO_4 = 11·6 per cent. N.

Nitrogen Distribution (Hausmann Numbers).

A preliminary estimation was made of preparation *B. coli* 135. 0·2030 grm. of dried alcohol-soluble protein was digested in a flask with reflex condenser with 50 c.c. 20 per cent. HCl, and the process followed according to Plimmer's (8) description.

The final results, which are tentative, were as follows :—

Amide N. .. .. = 3·70
Diammo N. .. .. = 2·70
Mono-Ammo N. by difference .. .. .. = 4·8
Total N. .. .. = 11·1

The amide nitrogen was determined by Thimann's (9) method in preparation 222. *S. aureus*, 0·2736 grm. dried alcohol-soluble protein, was hydrolised with 20 per cent. H_2SO_4, the hydrolysate diluted to 200 c.c.

Total N was determined in 20 c.c. portions.
Average 2·5 c.c. N/10 H_2SO_4 = 11·5 per cent. N.
Amide N determined in two 20 c.c. portions.
Average 2·2 c.c. N/50 H_2SO_4 = 2·27 per cent. N.

One examination for glutaminic acid was made on preparation 216, *S. aureus*. Dried alcohol-soluble protein, 0·100 grm., was hydrolised with strong HCl, concentrated *in vacuo* and saturated with dry HCl gas, cooled to 0° C. for 24 hours. A crop of white crystals separated, which microscopically resembled glutamic acid ; the yield was small and only 2 mgs. were obtained.

The general tests, solubility in alcohol of 70 to 80 per cent., and the preliminary nitrogen estimations, are evidence of a protein of prolamin type. This substance appears in all the varieties of bacteria examined—*Staphylococcus aureus*, *Streptococcus fæcalis*, *Streptococcus pyogenes*, a diphtheroid bacillus of the type of *B. Hoffmann*, *B. coli communis*, *B. paracoloides*, and *B. typhosus*. The amounts present in various types of bacteria appear to vary, and it is not yet possible to say if the alcohol-soluble proteins extracted from different species have different characteristics, but there is some evidence that such is the case. The alcohol-soluble protein is freely soluble in dilute alkali: thus dilute alkaline saline removes practically all the alcohol-soluble substance if alkaline saline is used instead of normal saline extraction before extracting with 70 per cent. alcohol; it is not soluble in dilute hydrochloric acid.

The final residue after removal of the alcohol-soluble protein is freely soluble in dilute alkali (0·2 per cent. KOH) and is precipitated with 10 per cent. acetic acid, added cautiously to maximum precipitation, as a bulky white precipitate. This substance gives all the usual protein reactions, including the glyoxylic, but the sulphur reaction (boiling with caustic alkali and lead acetate) is weak

This substance, which may be provisionally termed "residual protein," has been generally assumed to be a nucleo-protein (Heidelberger and Avery (5), Zinsser (10), etc.); however, repeated attempts to obtain purin bodies have given a uniformly negative result. The substance will form the subject of a further communication.

Injection of the various types of bacterial proteins has not so far proved pathogenic for rabbits—intravenously, subcutaneously, and interperitoneally—but anaphylactic phenomena have been observed.

Conclusions.—By a method which is shown to produce a minimum change in the protein constituents, an alcohol-soluble protein, having many of the characters of the alcohol-soluble proteins of the seeds of cereals, has been extracted from Streptococci, Staphylococci, *Bacillus Hoffmann*, *B. typhosus*, *B. coli communis*, *B. paracoloides*, and *Micrococcus catarrhalis*. The Molisch-reacting substance appears to form an important constituent of bacterial structure.

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Absence of a Linear Relationship between Graded Simple Reflex Flexions and the Relaxations thereof evoked by a Constant Extension-Producing Stimulus.

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(Received July 13, 1927.)

(From the Physiology Institute, Cardiff.)

When either of two antagonistic simple reflexes is evoked, its corresponding muscle shortens and any precurrent shortening in the antagonistic muscle may exhibit inhibitory lengthening. A flexion-producing stimulus evokes shortening in the flexor muscle and inhibitory relaxation of any precurrent extensor tone; an extension-producing stimulus evokes shortening of the extensor muscle and usually no phenomenon whatsoever in the flexor muscle—as flexor tone rarely occurs.

The concurrent application of these two stimuli evokes a compound reflex. The effect is Sherrington's "algebraic summation." Each muscle shortens—but the amount of shortening of a muscle in the compound reflex is smaller than its shortening in the corresponding simple reflex. The compound magnitude of shortening in either muscle is supposed to be conditioned by the algebraic summation, in the spinal centres, of the "excitation" produced by one of the stimuli and the "inhibition" produced by the other.

To the best of the writer's knowledge, the reflex magnitudes in this phenomenon have never been subjected to analysis and comparison. The object of the present paper is to examine one particular magnitude relationship—namely, that between the shortening (contraction) of a flexor muscle in a simple flexion-reflex and the lengthening (relaxation) produced in that muscle by the addition of an extension-producing stimulus, where the flexion-reflex is varied in magnitude and the extension-producing stimulus is kept at a constant intensity.

Methods.

The reflex phenomena treated in this paper are obtained in decerebrate cats. The isotonic method of registration is used. The excursions of the recording levers are measured at the same set time-point in the period of the simple or compound reflex. The magnitudes thus obtained are then compared.

The Special Conditions.

The flexion-producing stimulus is applied to the foot of the same limb as that of the recording muscle, and is either increased in intensity or decreased in intensity at each step in the experiment. An extension-producing stimulus is applied to the opposite foot and is kept at a constant intensity throughout the experiment. At each intensity of the flexion-producing stimulus it is first given alone, and the shortening of the flexor muscle (tibialis anticus) in the simple flexion-reflex thus evoked is later measured. The same stimulus is then given concurrently with the constant extension-producing stimulus and the reflex shortening of the flexor muscle is again measured. The point of measurement selected in each case is a time-point occurring at a selected fixed interval of time (1·0 second, 2·0 second, etc.) after the commencement of the simple stimulus or of the period of double stimulation. The constant extension-producing stimulus is usually allowed to run for two or three seconds before the commencement of the flexion-producing stimulus which is compounded with it. This general procedure is repeated for each of a number of different intensities of the flexion-producing stimulus. The measurements, of course, are made after completion of the experiment.

Under these conditions data are obtained concerning the reflex lengthening evoked by a constant extension-producing stimulus when acting against different magnitudes of simple reflex shortening of the flexor muscle. There are obviously many different possibilities. The lengthenings evoked by the constant extension-producing stimulus might be of equal magnitudes; or they might be equal proportions of the simple flexor shortening; and so forth.

Results.

If a series of three different magnitudes be

$$a_1, \qquad a_2, \qquad a_3,$$

and another series of three other related magnitudes be

$$a'_1, \qquad a'_3, \qquad a'_2,$$

the relationship between the two series will be linear if the difference between any two magnitudes in the first series and the difference between the two corresponding related magnitudes in the second series exhibit direct proportionality. That is, if

$$\frac{a_1 - a_2}{a'_1 - a'_2} = \frac{a_2 - a_3}{a'_2 - a'_3} = \text{C}.$$

In order to test the possibility of this relationship, the measurements of a graded series of simple flexion-reflexes are arranged in order of magnitude. The difference between each pair of these magnitudes is then found ("simple" grades). The magnitudes to which each of those simple flexion-reflexes is reduced by the action of a constant extension-producing stimulus ("inhibitory") are then put down in the order of their respective *simple* flexor shortenings. The differences between each consecutive pair of reduced magnitudes are calculated ("compound" grades). The magnitudes thus calculated for the "simple" grades if divided by the magnitudes calculated for the corresponding "compound" grades should give a constant, if the relationship between the simple flexor shortenings and either the magnitude by which each is reduced, or the magnitude which remains after reduction, is linear. Table I gives figures for one such experiment.

Table I.

Temporal order.	(i)	(ii)	(iii)	(iv)	(v)
	"Simple."		"Compound."		Ratio—A : B.
	Shortening.	Difference, A.	Shortening.	Difference, B.	
	mm. (× 2).	mm. (× 2).	mm. (× 2).	mm. (× 2).	
1	0·0		1·0		
		14·5		0·0 ?	∞
2	14·5		0·25		
		4·5		4·75	0·95
3	19·0		5·0		
		2·5		7·25	0·34
4	21·5		12·25		
		2·75		4·75	0·58
5	24·25		17·0		
		1·5		3·5	0·43
6	25·75		20·5		
		1·25		6·0	0·21
7	27·0		26·5		

Decerebrate cat: 4 hours 7 minutes after decerebration. One set of magnitudes between 6 and 7 (simple flexor shortening = 26·75 mm., compound = 19·5 mm.) is omitted. Description in text. [609 : 15386.]

The figures on the left of the table give the order of the simple flexor shortenings in ascending magnitude, and this order is also the temporal order of the observations in the experiment. In column (i) the magnitudes of flexor shortening (in millimetres as × 2 by the recording levers) are given for an ascending series of simple flexion-reflexes. In column (ii) the difference between each consecutive pair of these simple flexor shortenings is given. In column (iii) the remaining shortening of the flexor when the simple shortening in column (i) is acted against by a constant extension-producing stimulus is given. In column (iv) the difference between each consecutive pair of compound magnitudes in column (iii) is given. In column (v) each magnitude in column (ii) is divided by the corresponding magnitude in column (iv) to give a ratio.

It will be observed that the ratio given in column (v) diminishes markedly from above downwards—being infinitely large at the top of the column and comparatively small at the foot. There is, therefore, no linear relationship between the magnitudes of simple and of compound flexor shortening in this experiment.

For the further illustration of this experiment, the magnitudes are plotted graphically in fig. 1. The method adopted is to plot each of the simple flexor magnitudes upon a straight line which cuts the axis of abscissæ at an angle of 45°. Magnitudes of flexor shortening are measured along the ordinates of the graph, and the corresponding magnitude of compound flexor shortening is placed, at its proper distance from the axis of abscissæ, on a perpendicular line between that axis and the corresponding *simple* flexor magnitude. The compound flexor magnitudes are then joined together (by the dotted line). The dotted line gives an approximate indication of a curve representing the relationship of compound flexor shortening (or the relaxation of simple flexor shortening) to simple flexor shortening, when different magnitudes of the latter are plotted linearly.

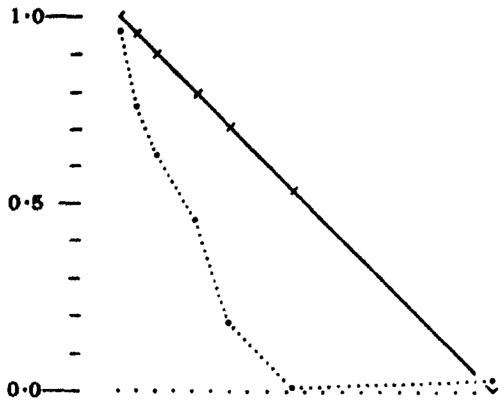

Fig. 1.

This experiment has been selected for illustration because it gives three salient points in reference to the relationships of the magnitudes of simple and of compound flexor shortening, and because it gives them comparatively free from possible experimental error. The first of these points is that where simple flexor shortening is maximal (or nearly so), the inhibitory relaxation produced by a certain extension-producing stimulus is small in magnitude or may not exist at all. The second salient point is that where the simple flexion is of about half maximal magnitude, the same extension-producing stimulus may give an inhibitory relaxation many times greater than that produced when it acts against a maximal flexion reflex. The third salient point is that, where the simple flexion is of very small magnitude, the same extension-producing stimulus may yet fail to produce complete inhibitory relaxation. If the relationship between the simple and the compound flexor shortenings is a linear one, all magnitudes of simple flexion, which themselves are smaller than the inhibitory relaxation of a medium-sized flexion-reflex, should exhibit complete inhibitory relaxation when opposed by the constant extension-producing stimulus. This possibility is absolutely negatived by the illustrative experiment. In it, a subliminal flexion-producing stimulus gives no trace of flexor shortening when applied alone; but when applied against a constant extension background, a small "compound" flexor shortening is evoked. This small shortening must be a physiological effect. It cannot be due to such an extraneous factor as "spread" of stimulating current—because no such shortening occurs when the flexion-producing stimulus is applied alone, and shortening should then occur if due to "spread."

In further illustration of this relationship, another example may be quoted (Table II and fig. 2). This experiment differs from the former in that the varied flexion-producing stimulus acted as the "background" during which a constant extension-producing stimulus was applied. In this case the "simple,"

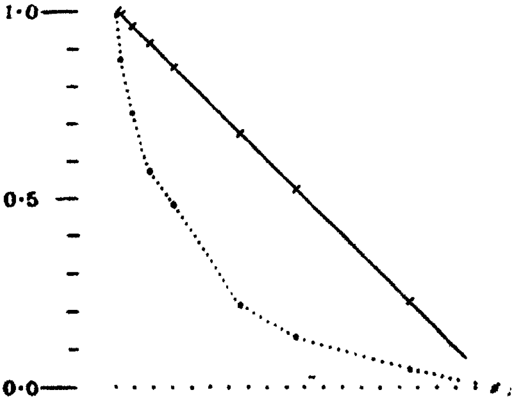

FIG. 2.

flexion-producing stimulus has evoked a certain amount of flexor shortening at the moment when the extension-producing stimulus is added. During the ensuing period (when the two stimuli are acting against each other), the simple flexor shortening partially lengthens—leaving a remainder of compound flexor shortening. Where the simple flexion is of small magnitude, this relaxation is complicated by such factors as the internal resistance of the shortened muscle to change in its own shape. In the experiment now quoted this factor possibly affected the first reaction in the series. If so, the residual flexor shortening in the compound reflex was smaller than actually appeared.

Table II and fig. 2 are exactly similar to the preceding table and figure, and the description for one serves for the other; the only difference is that the temporal order of the observations was reversed in the second experiment.

Table II.

Reverse temporal order.	(i)	(ii)	(iii)	(iv)	(v)
	"Simple."		"Compound."		Ratio— A : B.
	Shortening.	Difference, A.	Shortening.	Difference, B.	
	mm. (× 2).	mm. (× 2).	mm. (× 2).	mm. (× 2).	
1	0·0		0·0		
		10·5		2·25	4·67
2	10·5		2·25		
		14·0		3·75	3·74
3	24·5		6·0		
		7·0		4·0	1·75
4	31·5		10·0		
		8·0		12·5	0·64
5	39·5		22·5		
		3·0		4·0	0·75
6	42·5		26·5		
		2·0		7·5	0·27
7	44·5		34·0		
		1·5		6·5	0·23
8	46·0		40·5		
		0·5		5·5	0·09
9	46·5		46·0		

Decerebrate cat: 10 hours 57 minutes after decerebration. All grades of simple flexor shortening obtained in this experiment are included, but one set of magnitudes equivalent to 8 (simple flexor shortening, 46·0 mm.; compound, 41·5 mm.) is omitted. Description in text. [571 : 13994.]

General Observations.

Some points may be noted in connection with this relationship between magnitudes of simple and compound flexor shortening. In the first place, the observations in this paper have been confined to instances in which the compound shortening is of smaller magnitude than that of simple shortening, *i.e.*, to cases in which the compound effect is one of flexor relaxation. In many experiments no such inhibitory lengthening takes place in the whole range of flexor magnitudes; or, if it occurs at all, it is slight. Where this is the case, the relationship between simple and compound flexor shortening at different intensities of the flexion-producing stimulus (acted against by a constant extension-producing stimulus) is one of approximate equality, *i.e.*, the relationship between the two is approximately linear. In many experiments the compound flexor shortening is actually greater than the simple—flexor "augmentation" then occurs. In these cases, compound flexor shortening again deviates from a linear relationship with the simple flexor shortening.

In the second place, a point emerges from the curves which illustrate this present paper. In fig. 1 a comparatively sharp change of direction occurs in the curve of compound flexor shortening at reactions 2 (from the right-hand side). In fig. 2 the corresponding curve is much more smoothly graded. A similar sharp change of direction has been observed in some other experiments, but not in all. On the other hand, the sharp change in direction at this point certainly suggests the possibility that there may be two factors governing the resultant of the inhibitory effect of a constant extension-producing stimulus acting against varying flexion-producing stimuli. On the other hand, in this case the compound phenomenon exhibited by the antagonistic extensor muscle showed graded inhibitory lengthening at reactions 1, 2 and 3 (from the right-hand side). This suggests the possibility that the corresponding flexor contraction was accidentally depressed at reaction 2, and that the sharpness of change of direction in the curve there is more or less accidental.

In the third place, the graphs which illustrate this paper show comparatively simple relationship between the linear arrangement of simple flexor shortenings and the curve of compound flexor shortenings. In some cases the shape of the latter curve has been more sigmoid. On the left-hand side of the graph—where simple flexor magnitudes are approaching maximal—the compound flexor shortening is sometimes *greater* than the simple. Flexor augmentation occurs, but this augmentation—the difference between simple and compound flexor shortening—becomes less as maximum is approached.

In the fourth place, the results of this paper appear to negative a linear relationship between simple and compound reflex flexor shortening. Linear relationship includes generically many possible more specific relationships of the two magnitudes. Amongst such specific linear relationships are those of direct proportionality between the two magnitudes and of constant difference. Both these possible relationships are excluded by the general negation shown in this paper.

Summary.

1. In decerebrate cats, graded reflex magnitudes of simple flexor shortening are obtained in response to flexion-producing stimuli of different intensities. Each of these simple shortenings is then subjected to the "inhibitory" effect of a constant extension-producing stimulus—thus giving compound flexor shortening.

2. Comparison of the magnitudes of simple flexor shortening and of compound flexor shortening fails to establish a linear relationship between the two.

The expenses of this research were defrayed in part by grants from the Government Grants Committee of the Royal Society and from the Medical Research Council.

Absence of a Linear Relationship between the Reflex Flexor Shortenings evoked by a Graded Series of Flexion-Producing Stimuli and the "Inhibitory" Lengthenings of a Constant Extension Reflex evoked by the same Stimuli.

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(Received July 13, 1927.)

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When each of a graded series of flexion-producing stimuli is compounded with a constant extension-producing stimulus, a common reflex effect is that both of two antagonistic muscles—a flexor and an extensor—exhibit a smaller magnitude of shortening in the compound reflex than in their respective simple reflexes. Such a series of compound reflexes affords the opportunity of analysing various possible relationships. One relationship—that between the "simple" and the "compound" flexor shortening—was examined in a preceding paper.* The reverse aspect of this particular relationship is obtained by contrasting each different magnitude of "simple" flexor shortening with the corresponding magnitude of concurrent lengthening ("relaxation") in the extensor muscle. The present paper is devoted to an examination of this particular relationship.

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* 'Roy. Soc. Proc.,' B, vol. 102, p. 143 (above).

The relationship of flexor shortening and concurrent extensor lengthening is that which has chiefly been used by Sherrington for the establishment of the principle of the "reciprocal innervation of antagonistic muscles." Notwithstanding the interest and importance of this relationship, the reflex magnitudes involved have not hitherto been examined. All examinations have been more or less qualitative, and any theoretical illustrations—by the giving of hypothetical numerical values to flexion and extension (including, in each case, "excitation" and "inhibition")—have been made on the assumption of equality between "excitation" and "inhibition." The writer* has, however, shown that one particular reflex phenomenon—the concurrence in compound reflexes of a remainder of shortening in *both* of the antagonistic muscles—is inexplicable on the assumption that there is equality between "excitation" and "inhibition."

The proof given by the writer in the paper quoted above that "excitation" and "inhibition" are unequal does not itself negative the possibility of a relationship of simple proportionality between the two. Turning from theory to fact, the problem to be dealt with in this present paper is the possible occurrence of a relationship of simple proportionality between the simple "excitatory" shortening of the flexor muscle in the flexion-reflex and the concurrent "inhibitory" lengthening of an extensor muscle when the flexion is compounded against an extension-reflex. But that relationship of simple proportionality is only one of the possible linear relationships between series of these magnitudes; and the present paper deals with the wider issue—that of linear relationship in general.

The reflex phenomena here analysed are obtained in decerebrate cats by the isotonic method of registration. Synchronous records of the reflex shortenings and lengthenings of two antagonistic muscles—tibialis anticus and gastrocnemius-soleus are measured at set time-points in the period of the simple or compound reflex. The magnitudes thus obtained are then compared.

A New Method of Direct Comparison of Reflex Magnitudes in Antagonistic Muscles.

There is probably a comparatively simple explanation for the fact that comparison of reflex magnitudes in antagonistic muscles has scarcely yet been made.

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* 'Quar. Journ. of Exp. Physiol.,' vol. 14, p. 1 (1924).

Extensor and flexor muscles may (and do) act at different distances from the fulcrum of their joint—so that their shortenings necessary to produce equal (but opposite) angular movement at the joint are of different magnitudes. Further complications are produced by the differing mechanical magnifications of the recording apparatus used in experiment—it is, for instance, convenient to magnify extensor movements more greatly than flexor. The relative differences in reflex movements of flexor and extensor muscles could no doubt be brought into direct comparison by calculating the distances at which the respective muscles act from the fulcrum of the joint, but this measurement would be difficult to make and probably inexact.

For some years the writer has used a method of direct comparison for which the claim may perhaps be made that it affords a new method of research in this field. The method itself is simple. *Maximal* reflex shortenings of the flexor muscle in the flexion-reflex and of the antagonistic extensor muscle in the extension-reflex are evoked, recorded, and measured. All reflex magnitudes in either muscle are calculated as ratios of the maximum reflex shortening of that muscle. Direct comparison is made between these ratios. This method, by one simple calculation, adjusts all the differences due to the method of recording, to the constitution of the muscles themselves, and to their mechanical arrangement.

The magnitudes thus obtained may be treated in several different ways. Of these, one is the mode of calculating proportional relationships between different magnitudes. In some cases (as where the relationships all occur in the same muscle) it is not necessary to calculate an intermediate ratio of maximum shortening; the direct magnitudes of the excursions of the recording levers may be used. In other cases (as where the magnitudes of antagonistic muscles are to be compared) it is sometimes necessary to use ratios of maximal shortening—the investigated ratio is therefore then a ratio of two ratios.

A second and useful mode of treatment is the graphic—the plotting of relative magnitudes (or ratios) on one and the same graph. For this purpose again a new method has been used. One selected series of reflex magnitudes is plotted upon a straight line which cuts the axes of abscissæ and ordinates in a graph at angles of 45° (or at other convenient angles). In reality, such a graph plots reflex magnitudes both along the abscissæ and along the ordinates. The ordinates are selected for the plotting, and all other magnitudes corresponding to the member of the linear series which falls upon a particular ordinate are placed upon that ordinate. If a curve is drawn through these other magnitudes its shape in contrast with the linearity of the selected magnitudes is seen at a glance. All reflex magnitudes (or ratios) from two antagonistic muscles are sometimes plotted upon the same graph. On other occasions a double graph is used for the relative magnitudes in flexor and extensor muscles. In this case the two graphs have a common axis of abscissæ, but flexor magnitudes are plotted above the zero line and extensor magnitudes are plotted, in the opposite direction, below.

The writer* has already made use of these methods for the direct comparison of reflex magnitudes ; namely, the method of direct comparison of magnitudes in terms of the ratios of maximal reflex shortening, and the method of graphic plotting of one series of such ratios upon a sloping straight line, and of other ratios related to a member of the series upon the proper corresponding ordinate.

The Special Conditions.

The magnitudes used in this present analysis are obtainable from the same kinds of series of reflex phenomena as those described in a previous paper.† The magnitudes of simple flexor shortening evoked by a graded series of flexion-producing stimuli are measured as before. The same stimuli are then applied concurrently with a constant extension-producing stimulus—and usually two or three seconds after the commencement of that stimulus. The effect of the addition of the flexion-producing stimulus is to reduce the reflex shortening of the extensor muscle which is in being at the time of the application of the stimulus. "Test" simple extension-reflexes are recorded from time to time, and—a fixed time-point having been selected—the magnitude of inhibitory lengthening ("relaxation") in the extensor muscle is measured. These measurements give data concerning the relation between different magnitudes of simple reflex shortening of the flexor muscle and each corresponding magnitude of compound reflex lengthening of a constant extension-reflex.

Results.

Let the magnitudes of flexor shortening evoked by flexion-producing stimuli of three different intensities be

$$a_1, \quad a_2, \quad a_3,$$

and let the magnitudes of lengthening of a constant extension-reflex produced by each of those three stimuli be

$$R_1, \quad R_2, \quad R_3,$$

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* 'Quar. Journ. Exp. Physiol.,' vol. 14, p. 1 (1924)—see fig. 5 on p. 16.

† 'Roy. Soc. Proc.,' B, vol. 102, p. 143 (above).

the relationship between the two series of magnitudes will be linear if the differences between any two magnitudes in the first series and the differences between the corresponding two magnitudes in the second series exhibit direct proportionality. That is :—

$$\frac{a_1 - a_2}{R_1 - R_2} = \frac{a_2 - a_3}{R_2 - R_3} = C.$$

Tables I, II and III give calculations of this ratio for different intensities of the flexion-producing stimulus in three different experiments. The figures at the left-hand of each table give the numbers of the observations in order of time and also in order of ascending magnitude. In column (i) magnitudes of simple flexor shortening are given in millimetres (as × 2 by the recording levers). In column (ii) the difference between each consecutive pair of these simple flexor shortenings is given. In column (iii) are given (in millimetres as × 4 by the recording levers) the magnitude of lengthening of the extensor muscle in a constant extension-reflex acted against by the flexion-producing stimulus which gives the corresponding flexor shortening in column (i). Column (iv) gives the difference in each consecutive pair of these compound extensor lengthenings. Column (v) gives the ratio obtained by dividing the magnitude in column (ii) by that in column (iv). It will be observed that this ratio varies greatly.

Table I.

Temporal order.	(i)	(ii)	(iii)	(iv)	(v)
	" Simple " flexor.		" Compound " extensor.		Ratio— A : B.
	Shortening.	Difference, A.	Lengthening.	Difference, B.	
	mm. (× 2).	mm. (× 2).	mm. (× 4).	mm. (× 4).	
1	0·0		1·0		
		4·25		1·9	2·24
2	4·25		2·9		
		1·0		3·85	0·26
3	5·25		6·75		
		2·5		13·25	0·19
4	7·75		20·0		

Decerebrate cat: 25 hours 16 minutes after decerebration. All grades of simple flexor shortening obtained in this experiment are included, but two sets of magnitudes obtained with subliminal flexion-producing stimuli are omitted. Description in text. [663 : 17271.]

Table II.

Temporal order.	(i)	(ii)	(iii)	(iv)	(v)
	"Simple" flexor.		"Compound" extensor.		Ratio—A : B.
	Shortening.	Difference, A.	Lengthening.	Difference, B.	
	mm. (× 2).	mm. (× 2).	mm. (× 4).	mm. (× 4).	
1	0·0		2·0		
		15·5		8·25	1·77
2	15·5		10·75		
		4·5		8·0	0·56
3	20·0		18·75		
		3·5		5·5	0·64
4	23·5		24·25		
		3·0		0·75	4·00
5	26·5		25·0		

Decerebrate cat: 4 hours 7 minutes after decerebration. The magnitudes given were obtained consecutively in the course of the experiment. One set of magnitudes obtained with a subliminal intensity of flexion-producing stimulus before the commencement of this series, and three sets of magnitudes at which complete extensor lengthening occurred after the end of the given series, are omitted. Description in text. [609 : 15386.]

Table III.

Temporal order.	(i)	(ii)	(iii)	(iv)	(v)
	"Simple" flexor.		"Compound" extensor.		Ratio—A : B.
	Shortening.	Difference, A.	Lengthening.	Difference, B.	
	mm. (× 2).	mm. (× 2).	mm. (× 4).	mm. (× 4).	
1	0·0		1·5		
		1·0		0·5	2·00
2	1·0		2·0		
		4·5		0·0	∞
3	5·5		2·0		
		20·0		1·0	20·00
4	25·5		3·0		
		5·5		1·0	5·50
5	31·0		4·0		
		4·5		1·5	3·00
6	35·5		5·5		
		2·0		8·0	0·25
7	37·5		13·5		
		2·5		4·5	0·56
8	40·0		18·0		
		3·0		1·0	3·00
9	43·0		19·0		
		2·0		0·0	∞
10	45·0		19·0		

Decerebrate cat: 5 hours 38 minutes after decerebration. The observations up to No. 7 were obtained consecutively in the experiment; one observation between 7 and 8 (where the magnitudes were almost identical for those given for 8) and one observation between 8 and 9 (where the magnitudes were almost identical with those given for 9) are omitted. Description in text. [570 : 13886.]

Figs. 1, 2 and 3 give graphic representations of the magnitudes (as ratios of maximum shortening) in the corresponding tables. In each case proportional magnitude of simple flexor shortening is plotted upon a straight line which cuts the axis of abscissæ at an angle of 45°. Ordinates measure the magnitude of shortening or lengthening calculated as a ratio of maximal reflex shortening in flexor or extensor muscle. On the same ordinate as simple flexor shortening, the corresponding compound extensor lengthening is plotted. A broken line joins up these extensor magnitudes. [The scale in fig. 1 is twice that in the two remaining figures.]

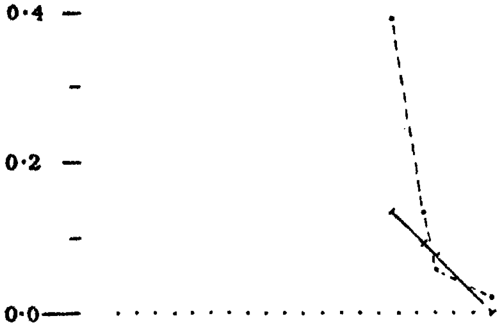

Fig. 1.

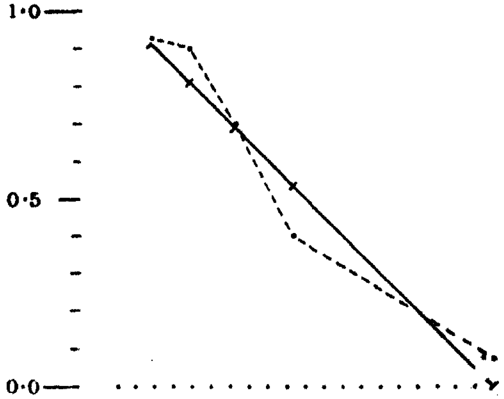

Fig. 2.

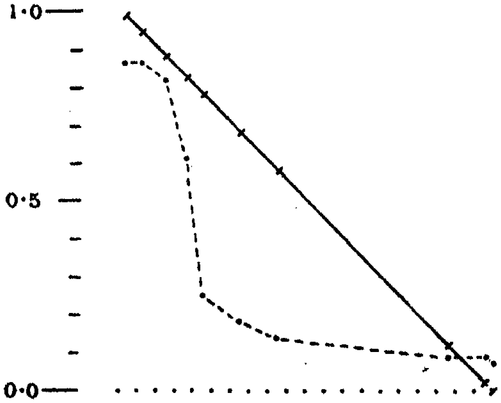

Fig. 3.

From Table I and fig. 1 it is seen that the flexor magnitudes of this particular experiment are all comparatively small. The flexor magnitudes lie in the lower part of the scale. Had the series been extended in an upward direction, it is probable that nearly maximal extensor lengthening would have occurred at or before the corresponding simple flexion was of about half maximal size. This is a very common relationship of the two phenomena. And the illustration gives, as it were, a portion only of the graphs of the two phenomena.

Table II and fig. 2 give an instance in which there is greater correspondence between the two magnitudes. From the table it will be observed that the ratio decreases to a minimum and again rises as the magnitude of simple flexor shortening progressively increases. This form of variation in the ratios is of extremely common occurrence, and signifies a more or less sigmoid form in the curve of compound extensor lengthening when it is plotted against a linear arrangement of simple flexor shortenings.

Table III and fig. 3 illustrate an example in which the sigmoid form of the curve of extensor lengthening is evident. In this case the extensor lengthening increases rapidly only after the simple flexor shortening has exceeded about 0·75 of maximal. The general form of this curve of extensor lengthening is similar to that obtained in the more common instances where nearly maximal extensor lengthening is attained while the flexion-reflexes are still small. The form of the curve of the extensor lengthening here figured is a very usual one, but the relationship of the steepest part of the curve with large flexor shortenings is unusual. The ratios in the table again show a decline to a minimum and thereafter further increase as the magnitudes of simple flexor shortening steadily increase. There is again no approximation to a linear relationship between the series of magnitudes of simple flexor shortening and the series of magnitudes of compound extensor lengthening.

General Observations.

Examination of a large number of series of compound reflexes has shown that the phenomena illustrated in the three examples selected for this paper are of almost constant occurrence. Extensor lengthening does not bear a linear relationship to the simple flexor shortening evoked by the same flexion-producing stimulus which gives the lengthening when it acts against a constant extension-reflex.

Notwithstanding the generality of this statement, approximations to a linear relationship are occasionally observed. This appears to be the case where "inhibitory" lengthening of the *flexor* is absent, or is small, in the compound reflex. Where that flexor lengthening ("relaxation") is greatest, the deviation from linearity examined in this paper appears to be greatest. It may be that the linear relationship which is occasionally encountered lies intermediate between what may be described as positive and negative curvilinear relationships. Of these, the examples quoted in this paper belong to one type only.

The experimental observations in this paper, therefore, negative the possibility of a *general* linear relationship between series of simple flexor shortenings and the extensor lengthenings given by varied flexion-producing stimuli acting against a constant extension-producing stimulus. This negation includes two possible specific relationships of excitatory contraction (in the flexor muscle) and inhibitory relaxation (in the extensor muscle). The relationship between the former and the latter, *i.e.*, between simple flexor shortening and compound extensor lengthening, is not one in which the lengthening and shortening are related in direct proportionality. Nor is that relationship one in which there is a constant difference between the two magnitudes.

Summary.

1. In decerebrate cats, graded reflex magnitudes of simple flexor shortenings are obtained in response to flexion-producing stimuli of different intensities. Each of those stimuli is then compounded with a constant extension-producing stimulus, and the resultant lengthening (relaxation) of the extensor muscle is measured.

2. Comparison of the magnitudes of simple flexor shortening and of compound extensor lengthening fails to establish a general linear relationship between the two.

The expenses of this research were defrayed in part by grants from the Government Grants Committee of the Royal Society and from the Medical Research Council.

The Relation of the Magnitudes of Remaining Reflex Shortening in Two Antagonistic Muscles during Compound Stimulation.

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(Received July 13, 1927.)

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In a decerebrate cat, a flexion-producing stimulus evokes reflex shortening of a flexor muscle and " inhibitory " relaxation of extensor tone, *i.e.*, lengthening of the antagonistic extensor muscle. An extension-producing stimulus evokes reflex shortening in the extensor muscle of the antagonistic pair but, as there is no flexor tone, no measurable phenomena occur in the flexor muscle. If those two antagonistic stimuli are applied concurrently, a common phenomenon is the occurrence of some degree of shortening synchronously in both muscles of the antagonistic pair.

On theoretical ground it is supposed that a simple flexion-producing stimulus evokes increase of discharge in the motor neurones which serve flexor muscles and " inhibition " of any discharge occurring at the time in the motor neurones which serve the antagonistic extensor. Similarly, an extension-producing stimulus is supposed to give concurrent increase of discharge in extensor moto-neurones and " inhibition " in flexor moto-neurones. A compound reflex is evoked when both these stimuli are applied concurrently, and a common (but not invariable) observation is that neither of the muscles in question shortens so greatly in the compound reflex as it does in its proper simple reflex. If the compound phenomena are regarded from the aspect of the effect upon either simple reflex of compounding it with an antagonistic simple reflex, both muscles appear to relax (lengthen). Sherrington* has described this compound reflex effect as " algebraic summation." It has often been assumed, sometimes tacitly, that the " excitation " and " inhibition " produced by a reflex stimulus are equal, and that the compound effect of two antagonistic reflex stimuli is produced by the algebraic summation in either group of moto-neurones (*i.e.*, in either of what the writer has termed the antagonistic " half-centres ") of the " excitation " and " inhibition " produced in it by the two antagonistic reflex stimuli. A corollary to this is that the " reciprocal " relationship of two antagonistic muscles is governed by the " excitation " of one and the equal " inhibition " of the other evoked by a reflex stimulus.

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* 'Roy. Soc. Proc.,' B, vol. 86, pp. 219 and 233 (1913).

The occurrence of central inhibition is an inference from the observed fact that in many cases one of the pair of antagonistic muscles may relax (*i.e.*, lengthen) during reflex stimulation. It is undoubted that inhibition occurs and plays an essential part in simple and compound reflex phenomena. That inhibitory lengthening—the phenomenon from which central inhibition is inferred—is an *invariable* part of simple and compound reflex phenomena is not only open to challenge but fails in the test of experiment, even where there may be an ample " background " of shortening upon which the inhibition might act. In other words, the usually assumed rule of reflex innervation—that relaxation of one antagonistic muscle accompanies contraction of the other—while accurate in a large number of cases, is not a constant one.

Most of the observations of this phenomenon have up to the present been qualitative. The actual quantitative magnitudes of reflex shortening and lengthening have scarcely been examined. The writer* has, however, shown that the observed synchronous occurrence of some degree of reflex shortening in both of two antagonistic muscles during a compound stimulation is incompatible with the assumption that the "excitation" and "inhibition" evoked by a reflex stimulus are equal.

A method of experimental approach to these problems is to record a graded series of simple reflexes (flexion or extension), and to compound each of those graded reflexes with a constant antagonistic reflex (extension or flexion). The simple reflex magnitudes are arranged in a linear series and the compound phenomena corresponding to each member of the graded series of simple reflexes are examined for the presence of, or deviation from, linearity, *i.e.*, for the occurrence or non-occurrence of direct proportionality with the simple reflexes which evoke them. It has already been shown by the writer† that direct proportionality does not always obtain in two possible relationships—that between the gradation of a series of simple flexor shortenings and the flexor lengthenings evoked when each member of the series is compounded with a constant extension-reflex; and that between the simple flexor shortenings and the compound lengthenings of the antagonistic extensor in the same circumstances.

The object of the present paper is to describe a certain linear relationship which has been found.

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* 'Quar. Journ. Exp. Physiol.,' vol. 14, p. 1 (1924).

† 'Roy. Soc. Proc.,' B, vol. 102, pp. 143 and 150 (above).

Methods.

Decerebrate cats are used. A flexion-producing stimulus is applied to the foot of the same limb in which the reflex movements of a pair of antagonistic muscles (tibialis anticus and gastrocnemius-soleus) are recorded by isotonic mechanical registration. These reflex movements are measured. An extension-producing stimulus is applied to the opposite foot. The reflex movements in the original pair of antagonistic muscles are again recorded and measured. The two stimuli are applied concurrently. The reflex movements are again recorded and measured. This procedure is adopted for different arrangements (intensities) of the antagonistic stimuli. In some of the present observations, the flexion-producing stimulus is varied in intensity, and the extension-producing stimulus is kept constant, or *vice versa.* In other experiments the intensities of both stimuli are progressively increased at each step. The change in intensity of stimulation may be in the direction either of increase or of decrease.

The reflex magnitudes thus obtained are calculated as ratios of the respective maximal reflex shortening of the flexor or of the extensor muscle, as the case may be. More than 150 reflex series of this description have been recorded, measured, and examined. They were recorded at an average interval of a little more than 7 hours 20 minutes after decerebration ; but four of the series were recorded less than 2 hours after decerebration and 11 more were recorded less than 4 hours thereafter. Twenty-six on the reflex series were recorded between 10 and 28 hours after decerebration.

The phenomena selected for examination in this paper are the magnitudes of remaining shortening in the two antagonistic muscles during compound stimulation ; and, in particular, the sum of the two shortenings (as estimated as ratios of the corresponding maximal reflex shortenings of the respective muscles).

An Example of "Constancy of Sum" of the remaining Reflex Proportional Shortenings in two Antagonistic Muscles during Compound Stimulation.

A chief difficulty in this experimental analysis is to secure constancy of the reflex preparation. When such constancy is obtained, there still remain technical difficulties with regard to the calculation of the zero datum from which all reflex shortenings are measured, and with regard to the calculation of the magnitude of maximal reflex shortening. In one favourable example conditions of considerable constancy were obtained, and the zero and maximum data were obtainable with a close approximation to accuracy. The results to be described were obtained at 27 hours 31 minutes after decerebration.

Graded simple flexion-producing stimuli of progressively increasing intensities were applied and evoked simple flexion-reflexes of graded magnitudes. At each intensity the flexion-producing stimulus was also applied concurrently with a constant extension-producing stimulus—the point of application of the flexion-producing stimulus being in each case 3 seconds after the commencement of the "background" extension-producing stimulus. The two stimuli ran concurrently for a little more than 2 seconds of time and the reflex measurements given in the following table (Table I) are the various magnitudes of compound reflex shortening measured at 2 seconds after the commencement of the stimulation.

In Table I the figures in the left-hand column give the temporal order of a series of compound reflexes—and this order is also the order in which the intensity of the flexion-producing stimulus was increased. Column (i) gives the magnitudes of compound flexor shortening in millimetres (as × 2). Column (v) similarly gives the magnitudes of compound extensor shortening in millimetres (as × 4). Column (ii) gives the ratios of maximum flexor shortening corresponding to the magnitudes in column (i), and column (iv) similarly gives the ratios of maximal extensor shortening corresponding to the absolute magnitudes in column (v). In column (iii) the two ratios in columns (ii) and (iv) are summed.

Table I.

Temporal order.	(i)	(ii)	(iii)	(iv)	(v)
	Flexor.		**Sum.**	Extensor.	
	mm. (× 2) *a*.	Ratio. *b*.	Ratios. *b* + B.	Ratio. B.	mm. (× 4). A.
1	0·0	0·0	0·726	0·726	37·0
2	14·5	0·254	0·911	0·657	33·5
3	23·5	0·413	1·007	0·594	30 25
4	30·75	0·540	1·065	0·525	26·75
5	32·0	0·562	1·013	0·451	23·0
6	36·25	0·637	1·025	0·388	19·75
7	38·5	0·675	0·994	0·319	16·25
8	46·0	0·807	0·939	0·132	6·75
9	53·75	0·943	1·036	0·093	4·75
10	55·25	0·969	1·067	0·098	5·0
11	55·25	0·969	1·033	0·064	3·25
12	55·25	0·969	1·038	0·069	3·5
13	55·75	0·978	1·056	0·078	4·0

Flexor maximum = 57·0 mm.
Extensor maximum = 51·0 mm.

Decerebrate cat: 27 hours 31 minutes after decerebration. Description in text. [664: 7350.]

Except in the case of the first observation, both of the two antagonistic muscles exhibit reflex shortening during compound stimulation. There is a very close approximation to constancy in the sum of the ratios from observation 3 downwards, in spite of the fact that the possible range of experimental error scarcely justifies the giving of the ratios to the 1000th part. It will be observed that the magnitudes increase from above down in the case of the flexor muscle, and decrease from above down in the case of the extensor. Notwithstanding the wide variation in opposite directions of the flexor and extensor magnitudes, there is constancy in the sums of their ratios within a limit of variation which is remarkably small for observations of this nature. It is hard to resist the conclusion that, in this experiment, the sum of the ratios should have amounted exactly to unity; if so, the average deviation from unity in the observations from No. 3 down is less than 4 per cent. [This result may perhaps be held to indicate the general reliability of the methods here adopted for bringing flexor and extensor phenomena into direct comparison.]

This particular relationship of the remaining shortenings of two antagonistic muscles in compound reflexes (when these shortenings are calculated as ratios of reflex maxima) is one of "constancy of sum." The quoted example is the most striking which has been obtained, but approximations of this constancy have occurred in many reflex series and some of these have shown almost as great constancy as the selected example. A marked feature of the calculations has been the frequent occurrence of sums varying but slightly from unity. The sum is commonly (but by no means invariably) about 5 per cent. greater than unity, and this excess may perhaps be attributed to error in the calculation of the data—and most probably to underestimation of the flexor maximum datum. Occasionally the sum has been considerably in excess of unity. Sums as great as 1·3 have occasionally been met, and it is possible that the excess over unity may be attributed to underestimation of one or other maximum. The frequency, however, with which the sum approximates to unity completely negatives the possibility that this approximation is a chance phenomenon.

That the summed remainders of proportional reflex shortening should approximate to unity is not in opposition to the mechanics of movement at joints. Two antagonistic muscles which act upon the same joint pull upon a rigid lever, and act on either side of its fulcrum. The tendons by which they are connected to the bony lever are usually attached at different distances from the fulcrum. If the antagonistic muscles are controlled in such a way during reflex movements that their lengths are always accurately adjusted to the posture of the joint (*i.e.*, if on the one hand any "slack" is always taken up and on the other hand neither muscle tends to overpull the other), then when one muscle shortens by a certain proportion of its own maximal shortening, the other muscle must lengthen by that same proportion of *its* maximal shortening. Were this not so, the muscles would either pull against each other in a movement or one of them would remain slack.

Neither of the above possibilities is physiologically impossible, but this constancy of sum at unity seems to indicate that, in some reflex series at any rate, the lengths of the antagonistic muscles remain throughout nicely adjusted the one for the other. This is certainly the case in many instances, although not invariably so.

A point of importance is that owing to the conditions of the experiment the two antagonists are mechanically free from one another, so that the pull of one cannot mechanically affect the other. "Constancy of sum" might conceivably be brought about by an adjusting proprioceptive reflex stimulus which might compensate any tendency for one muscle to pull against the other. But constancy of sum here occurs where the two muscles are pulling upon separate levers (the writing levers of the recording apparatus), and it may be concluded that the relationship of constancy of sum (where it obtains) is a central phenomenon brought about in the spinal centres.

In the particular series from which the example in Table I was obtained the phenomenon of compound flexor relaxation (lengthening) was not well marked. This failure of the occurrence of flexor inhibitory lengthening is a common feature of the reflex series which have exhibited constancy of sum. But it is not an invariable feature; constancy of sum occurred in one of the series where flexor inhibitory lengthening was most marked of any. As regards reflex series where the flexion-producing stimulus is varied in intensity while the extension-producing stimulus is kept constant, constancy of sum has been associated with the selection of nearly maximal extension for the constant extension-reflex.

Exceptions to the "Constancy of Sum" Relationship.

Where the sum of the remainders of proportional shortening in two antagonistic muscles is constant, the series of these sums bears a linear relationship to the series of graded simple reflexes, or to the series of constant simple reflexes, which are compounded together. It may indeed be looked upon as a special instance of this linear relationship. While constancy of sum has occurred in many instances, it has been by no means invariable; and in some cases in which inconstancy has occurred there has yet been clear linear relationship between the series of sums and the graded series of simple reflexes used in the particular experiment. Fig. 1 gives one such instance in graphic form.

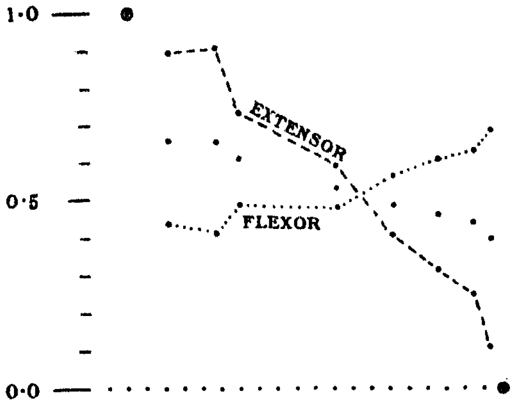

Fig. 1.—Decerebrate cat: 3 hours 46 minutes after decerebration. Reflex magnitudes (ratios) in left tibialis anticus and gastrocnemius-soleus at 2·0 seconds in the period of compound reflex stimulation. Extension-producing stimulus graded. Flexion-producing stimulus constant. Selected gradation of graph is that of simple flexor shortenings in *right* hind limb. Description in text. [530 : 11931.]

In the experiment here plotted, the extension-producing stimulus was varied in intensity, and the flexion-producing stimulus was constant. The remainders of shortening in the flexor and extensor muscles when the two stimuli were compounded are plotted together on the same graph above the axis of abscissæ —distance from the axis of abscissæ representing remainder of shortening calculated as a ratio of maximal reflex shortening. The extension-producing stimulus decreases in intensity from left to right, and the extensor remainders in the compound reflexes are joined up with broken lines. These remainders are plotted with ordinates corresponding to the straight gradient upon which the corresponding *simple* flexor shortenings in the *opposite* limb were plotted—and the maximum and minimum of this straight gradient are denoted by dots surrounded by circles. The flexor remainder corresponding to an extensor remainder is plotted on the same ordinate, and the flexor remainders are joined up by dotted lines. The mean of the sum of two corresponding flexor and extensor remainders is plotted as a dot upon the same ordinate, and these dots are not joined up. They lie approximately on a straight line.

Other examples of this linear relationship accompanying inconstancy of sum of proportional remainders have been obtained. Where the flexion-producing stimulus is that which is varied in intensity, the linear relationship rarely extends beyond the zero point of the flexor gradient, *i.e.*, this linear relationship becomes distorted where stimuli of subliminal intensity as regards simple shortening of the flexor muscle may yet evoke compound lengthening (relaxation) of the extensor muscle when pitted against a constant extension-producing stimulus.

The experiment from which the magnitudes in fig. 1 are taken had an additional point of interest. The sums of the remainders of shortening in a flexor and extensor of one hind limb show a clear relationship with the similar sums for the corresponding muscles of the other hind limb. The varied stimulus used in the experiment gave graded flexion in the right ankle flexor—that being the limb to which it was applied. The same stimuli gave graded simple shortening of the left extensor. Thus the stimulus pattern was diametrically opposite in the two hind limbs; in the right—graded flexion against constant extension, in the left—graded extension against constant flexion. The *difference* of the sums of the proportional remainders of shortening in both limbs at each grade of the varied stimulus gave a comparatively close approximation to equality. This is shown in Table II.

Table II.

Temporal order.	(i) Right.	(ii) Left.	(iii) Difference.
1	0·26	0·79	0·53
2	0·34	0·88	0·54
3	0·52	0·92	0·40
4	0·46	0·97	0·51
5	0·61	1·07	0·46
6	0·77	1·22	0·45
7	0·86	1·32	0·46
8	0·89	1·32	0·43

Decerebrate cat: 3 hours 46 minutes after decerebration. Description in text. [530 : 11931.]

The figures in the left-hand column give the order of a series of compound reflexes in temporal sequence, and this order is also the order of increasing intensity of a reflex stimulus applied to the right foot. Column (i) gives the sum of the proportional remainders in right extensor and flexor at each intensity of the graded stimulus, as measured at the 2-second time-point in the compound reflex. Column (ii) gives the sum of the corresponding remainders in the left extensor and flexor. Column (iii) gives the difference of those two sums. It will be observed that, while there is some variation in the differences, there is yet a considerable approximation to equality. This approximation is close enough when account is made for the four-fold possibility of error.

The particular linear relationship of the sums of remaining shortening in compound reflexes described in this section appears to occur where a simple reflex of small magnitude is selected as the constant reflex of a compound series in which the antagonistic reflex is of graded intensity.

In most of the reflex series so far examined there has been some approximation to a linear relationship between the sums of the remaining proportional shortenings and the magnitudes of the simple reflex shortening evoked by the graded stimuli used in such series; but curvilinear and irregular relationships have not infrequently been observed. The exceptions must be noted but cannot be analysed here. In some cases exceptional relationships appear to be associated with "deterioration" of the constant simple reflex used in a series—in other words, with inconstancy of the reflex preparation. In other cases it is possible that a curvilinear relationship is due to error in the estimation of the maximum datum. In yet other cases abnormality may be suspected—it occasionally looks as if inhibitory relaxation (lengthening) is unusually great in both muscles, and conversely it occasionally appears to be unusually small.

"Constancy of Sum" compared with "Reciprocal" Relationship.

The "reciprocal" relationship in reflex phenomena is that between reflex shortening of one muscle and reflex lengthening of its antagonist. The "constancy of sum" relationship described in this paper is that between the remainders of proportional shortening in both muscles irrespective of the occurrence of lengthening (relaxation) in one or other; and it is perhaps a special instance of a more general "linearity of sum" relation.

The "constancy of sum" relationship has occurred in experiments when the "reciprocal" relationship has been absent.

Where a graded series of flexion-producing stimuli is used, and each stimulus is compounded with a constant extension-producing stimulus, it not infrequently occurs that the *flexor* is more shortened in the compound reflex than in the simple reflex. In other words, no relaxation occurs—there is augmentation of contraction. Where this is the case it is clear that no "reciprocal" relationship obtains between extensor shortening (whether simple or compound) and flexor lengthening—for the latter is non-existent. Similarly, an extension-producing stimulus may evoke more *extensor* shortening when pitted concurrently against a flexion-producing stimulus than when applied alone. In this case there can be no reciprocal relationship between simple or compound flexor shortening and extensor lengthening (relaxation). Yet in each of those instances the relationship of constancy of sum may obtain between the remaining shortenings (estimated as ratios of maxima) in the two antagonistic muscles.

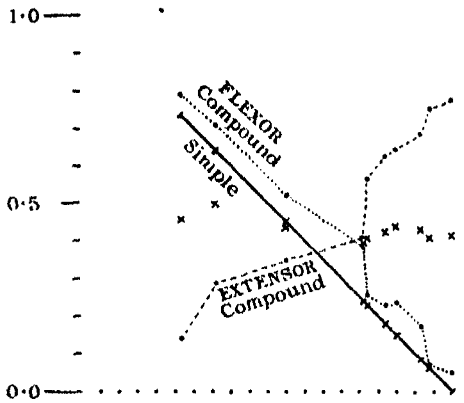

FIG. 2.—Decerebrate cat: 27 hours 31 minutes after decerebration. Reflex magnitudes in right tibialis anticus and gastrocnemius-soleus at 0·5 second in the period of simple or compound reflex stimulation. Flexion-producing stimulus graded. Extension-producing stimulus constant. Selected gradation of graph is that of simple flexor shortenings in the right hind limb. Description in text. [664 : 17350.]

In fig. 2 are plotted graphically the data from an experiment in which compound *flexor* augmentation of shortening occurred. It is the same experiment as that from which the magnitudes in Table I were obtained, but the proportional magnitudes here given are those at the 0·5 second time-point in the period of compound stimulation, and not those at 2·0 seconds. The same mode of graphical representation is adopted as that in fig. 1, save that the means of the sums of the proportional remainders are marked by small crosses. In addition, however, the simple flexor shortenings are plotted on a straight gradient represented by an unbroken line. The points representing proportional magnitudes of simple flexor shortening are marked by short cross lines placed at right angles to the continuous line. Had there been flexor lengthening in the compound reflex, the dotted line of flexor compound remainders would have fallen below the continuous line of simple flexor shortenings. It here lies everywhere above that line—in other words, there is compound flexor augmentation. Nevertheless, the "mirror image" relationship of the two series of flexor (dotted line) and extensor (broken line) remainders is very marked, and there is a comparatively close approximation to constancy in the mean of the sum of these remainders. The reciprocal relationship (as regards flexor relaxation) here fails, but constancy of sum obtains.

It may be noted parenthetically that fig. 2 in conjunction with Table I illustrates a feature of the constancy of sum relationship which occurs in well-marked instances. The method of treatment used in this paper has been, as it were, a static one. Reflex phenomena occurring at the same time-point in different reflexes are compared together. But the magnitudes of reflex movements are constantly varying during the period of one and the same simple or compound reflex. Table I and fig. 2 show that, in a particular experiment, constancy of sum occurred in a series of compound reflexes both at the 0·5 second time-point and at the 2·0 second time-point in the period of compound stimulation. As a matter of fact, approximations to constancy of sum appear to occur at each selected time-point; but the constant sums at the different selected time-points are not necessarily equal.

Increase of *extensor* shortening may also sometimes occur during compound stimulation. This is most usually the case where both the extension-producing and the flexion-producing stimuli are of low intensity. This phenomenon is often well illustrated where both of the antagonistic stimuli are progressively increased or decreased during the course of an experiment.

Fig. 3 gives the result of such an experiment in graphic form. In this case the graph is a double one; all flexor magnitudes (ratios of maximum) are plotted above the axis of abscissæ, and all extensor magnitudes (ratios) are plotted below the axis. The selected gradient is that upon which the simple flexor shortenings lie in a straight line. It is represented by the continuous line in the upper half of the graph, and the proportional magnitudes of simple flexor shortening obtained in the experiment are plotted at the points marked by short lines cutting the continuous line at right angles. All other simple and compound ratios are plotted on the corresponding ordinates upon which these simple flexor ratios fall. The dotted line in the upper part of the graph joins up the compound flexor remainders. The simple extensor proportional shortenings selected in this experiment to be pitted against the flexor are plotted in the lower half of the graph and are joined up by lines of dashes and dots. The broken line joins up the compound extensor remainders. The mean of the sum of each two remainders on an ordinate is plotted in the upper half of the graph only, and is marked by a cross.

The "parallelism" between the lines of flexor and extensor remainders is evident; and it will be observed that, with the exception of the second, the five crosses on the left-hand side lie upon a line which is approximately parallel to the axis of abscissæ, *i.e.*, the sums of proportional remainders of shortening are equal. Had there been constant extensor lengthening in the compound reflex, the line joining extensor remainders would always have lain between the axis of abscissæ and the line of simple extensor shortenings. At the fourth and

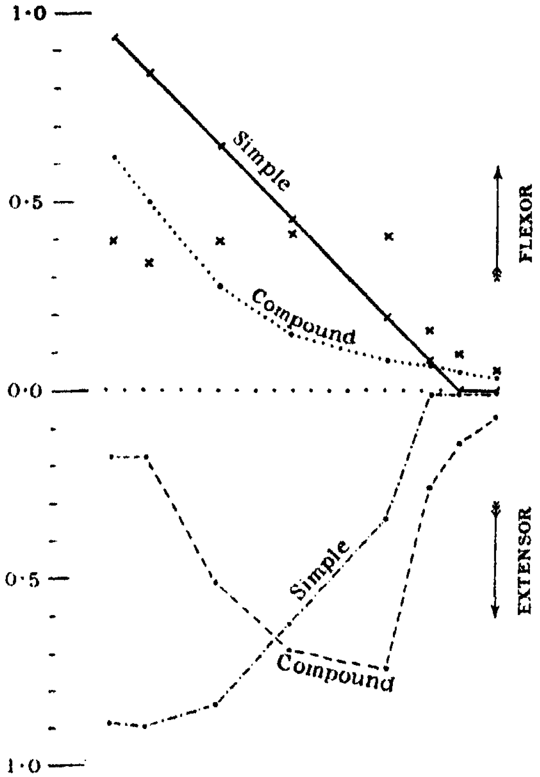

Fig. 3.—Decerebrate cat: 1 hour 25 minutes after decerebration. Reflex magnitudes in left tibialis anticus and gastrocnemius-soleus at 1·5 seconds in the period of simple or compound reflex stimulation. Both flexion- and extension-producing stimuli graded. Selected gradation of graph is that of simple flexor shortenings in left hind limb. In this graph extensor magnitudes are plotted below the axis of abscissæ and flexor magnitudes above. Description in text. [592 : 15060.]

fifth ordinates from the left of the graph the compound extensor shortenings fall below the positions of the simple flexor shortenings, and the same is the case with the remaining three compound extensor shortenings on the right-hand side of the graph. Here extensor *augmentation* of shortening occurs in the compound reflex but yet, at the fourth and fifth ordinates, the relationship of constancy of sum is retained. The reciprocal relationship as regards extensor lengthening and flexor shortening fails completely at these two ordinates although the relationship of constancy of sum in the proportional remainders obtains.

Comparatively close (and sometimes very close) approximations to the relationship of the simple and compound shortenings illustrated in fig. 3 have always been obtained where it has been possible to pit graded simple flexion-reflexes against graded simple extension-reflexes.

This phenomenon of extensor augmentation of shortening in compound reflexes raises questions of complexity. The experiments from which these illustrations have been taken have all been made in the decerebrate preparation of the cat. It might seem possible that the phenomenon may be due in part to the implication of the higher centres (mid-brain and so forth) still present in this preparation. But the phenomenon of extensor augmentation occurs in the muscles of the hind limb when the preparation is a low-spinal one.* It is also obtainable in the de-afferented state of the decerebrate cat,† *i.e.*, where the proprioceptive reflexes arising from the hind limbs are excluded. The inference is that the phenomenon is a part of the mechanism of the spinal centres.

Conclusions.

The reflex relationship described in this paper appears to open a field of some complexity. The observations are that in a large number of series of graded compound reflexes there is a definite relationship between the remaining shortenings in two antagonistic muscles. If these shortenings are measured as ratios of the corresponding maximal reflex shortening, the sum of the proportional remainders is constant in many experiments over a large portion of the reflex series. Not only so, but the constant sum often very closely approximates to unity. In other cases, however, where this sum shows inconstancy, there yet appears to be a linear relationship between the sums in question and the magnitudes of the graded series of simple reflex shortenings which are compounded against a constant reflex to give the compound reflexes.

The most inclusive statement of these relationships would appear to be defined as follows: If we call the sums of remaining reflex shortening in two antagonistic muscles Y, and the magnitude of simple reflex shortening evoked by the corresponding member of the graded series of simple reflex stimuli used in a compound series X, then

$$Y = AX + B,$$

where A and B are constants. In many cases (although not invariably) A appears to be zero, so that Y is itself constant. This defines a general linear relationship between the sums of remaining reflex shortenings and the magnitudes of simple reflex shortening evoked by the graded stimulus. Included in this linear relationship is one of constancy of sum of the remaining shortenings.

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* 'Quart. Journ. Exp. Physiol.,' vol. 7, p. 197 (1914)—see figs. 2 and 3.

† 'Quart. Journ. Exp. Physiol.,' vol. 7, p. 245 (1914)—see fig. 30.

The linear relationship appears to occur in the complete absence of the "reciprocal" phenomenon of inhibitory relaxation during compound stimulation. That is to say, where the linear relationship (and particularly the constancy of sum relationship) occurs in a series of compound reflexes, the "reciprocal" relationship may fail in part of that series. Where it fails, there is no inhibitory lengthening in one or other of the antagonistic muscles, but nevertheless the sums of the compound shortenings in these muscles are constant. To this extent it would therefore appear that the linear relationship between compound remainders is a more fundamental reflex phenomenon than the "reciprocal" relationship between shortening in one muscle and inhibitory lengthening in its antagonist.

The "reciprocal" relationship has chiefly been investigated in the case of the extensor muscle, *i.e.*, in the case of inhibitory relaxation (lengthening) of extensor tone or of extensor shortening in an extension reflex. The example (fig. 3) selected in this paper to illustrate an instance in which constancy of sum relationship obtained where there was no extensor lengthening in the compound reflex, is from an experiment in which it was possible to secure comparatively fine gradation in the series of simple extension-reflexes used against a graded series of simple flexion reflexes. In nearly every case in which this has been possible results approximating to those figured in this paper have been obtained. A peculiarity of the figure is that at the right-hand side of the graph—where both simple reflexes are of small magnitude—the extensor shortening in the compound reflex rises rapidly to a maximum. In this part of the curve there is inconstancy of sum accompanying ascending magnitudes of compound extensor shortening. The conditions for this type of result appear to occur where extensor tone is of small magnitude. If extensor tone is of large magnitude fine gradation of the simple extension-reflexes appears to be difficult to obtain. Under such conditions (not figured or described in this paper) the course of compound extensor shortening is different. At the right-hand side of the graph, where both of the antagonistic stimuli are of low intensity, subliminal flexion-producing stimuli give inhibitory lengthening of extensor tone; and this inhibitory lengthening increases in magnitude (and the resultant remainder of shortening decreases) as the intensity of the subliminal flexion-producing stimulus is increased up to and beyond the threshold of simple flexor shortening. As neither in the simple nor in the compound reflex does the flexor exhibit shortening, the remainder of extensor shortening is the only component in any sum relationship. In consequence, the sum of remaining shortenings progressively decreases. But at a certain point, where compound flexor shortening has appeared, constancy of sum of the two remainders occurs and is approximately retained.

It looks as if the result in either case is the attainment of constancy of sum of remaining reflex shortenings in the two antagonistic muscles; but that in one case (where initial extensor tone is low) this result is first attained (as the antagonistic reflex stimuli are progressively increased) by the augmentation in the shortening of the extensor muscle during compound stimulation; and that in the other case (where initial extensor tone is high) the same result is attained by inhibitory lengthening of the extensor in the compound reflex. If this is the case, the "reciprocal" phenomenon occurs where the initial tone is high, and is absent where it is low. In other words, the "reciprocal" phenomenon of inhibitory relaxation is only one of the two manners in which a possibly fundamental constancy of sum relationship is attained. The other manner is "irreciprocal"—augmentation of shortening in one or other of the antagonistic muscles during compound stimulation.

Summary.

1. Where two antagonistic reflex stimuli are applied concurrently, the remaining shortenings of two antagonistic muscles during compound stimulation are related to each other.

2. This relation is such that in many cases where one or both of the reflex stimuli are varied in intensity, the sum of the remaining shortenings in the two antagonistic muscles (each measured as a ratio to maximal reflex shortening) is constant.

3. In other instances this constancy fails, but a series of sums bears an approximate relationship of linearity to the magnitudes of the simple reflex shortenings evoked by the series of graded reflex stimuli which are used in the experiment. The most general statement of this relationship of sum is as follows: The sum of the proportional remaining shortenings in two antagonistic muscles, Y, is linearly related to the simple reflex shortening, X, evoked at each intensity of the series of graded reflex stimuli used in a series of compound reflexes, *i.e.*, $Y = AX + B$, where A and B are constants.

4. This relationship of the remaining shortenings in two antagonistic muscles during compound reflex stimulation appears to be a more fundamental one than the "reciprocal" reflex relationship between shortening in one muscle and inhibitory lengthening (relaxation) in its antagonist.

5. Notwithstanding the generality of these conclusions, exceptions to the linear relationship occur.

The expenses of this research were defrayed in part by grants from the Government Grants Committee of the Royal Society and from the Medical Research Council.

*Experiments on the Physiology and Genetics of the Smut Fungi.—Seedling Infection.**

By Sydney Dickinson.

(Communicated by Sir John Russell, F.R.S.—Received July 29, 1927.)

(From the Department of Mycology, Rothamsted Experimental Station, Harpenden, Herts.)

Introduction.

In a previous paper (1) the behaviour, on conjugation, of certain cultures of smuts was described. The origin of these cultures was as follows :—By means of the Dickinson Isolator (2) the first formed sporidium, arising from each of the four segments of the promycelium of a germinating smut chlamydospore (obtained from the infected ears of a known race of oats or barley) was isolated. Of the four cultures grown from the isolated sporidia of a single chlamydospore, two were found in every case to be of one gender (sex), and two of another gender. This gender distinction was determined by observing the behaviour of the hyphæ at the meeting of two colonies ; when the colonies were of different gender, the opposing hyphæ fused, whereas when the colonies were of the same gender the opposing hyphæ did not fuse. Up to the time of such conjugation the cells of the mycelium, hyphal or conidial according to the conditions, were found to be uninucleate. On the conjugation of two hyphæ a migration occurred of the nucleus and contents from one hypha into the other. Then followed the growth of a hypha called the "fusion-hypha," containing the two associated nuclei. Under certain conditions it was shown that the fusion-hypha gave off uninucleate hyphæ and conidia at either end, which resembled

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* This work was carried out with the aid of a grant from the Ministry of Agriculture and Fisheries.

the original (pre-conjugation) hyphæ in gender. The further history of the fusion-hypha will be described in a later paper. In the present paper experiments are described, in which various combinations of the above-mentioned genders are used in the inoculation of seedling plants.

Technique.

The seeds used were dehusked by hand, sterilised by steeping in formaldehyde, 1 : 320, for 4 hours, dried over night and germinated in sterile petri dishes. When the coleoptiles were about 5–10 mm. long, the seedlings were picked out with sterile forceps and laid on wet sand in earthenware dishes with covers.

The seedlings were inoculated after transference to the earthenware dishes. The inoculum was taken from tube cultures, a week to 10 days old, which were growing on 1 per cent. Lemco beef-extract agar.

The process of inoculation consisted in taking a small piece of mycelium from the edge of a culture and rubbing it over the surface of a coleoptile with a sterile platinum needle. The coleoptile was then moistened with sterile distilled water. If a second gender, either the same or different, was to be used in the inoculation, the whole process was repeated, the essential point being that the inoculum was not allowed to dry. Experiments were made using wound infection, and injection with a hypodermic syringe, but as these methods gave no better results, they were given up in favour of the simpler process.

Seedlings, two days after treatment, were stained in lactophenol and cotton blue. They were then slit into strips with a mounted needle and examined under a Leitz 2/3 objective followed by a Leitz 1/6 objective and a 10 × eyepiece. Each seedling was examined separately, and where no infection had occurred the smut hyphæ and conidia were seen to be growing superficially over the epidermis, while where infection had taken place the hyphæ could be traced from the surface through the epidermis and into the underlying tissues of the seedling.

Results and Conclusions.

Table I shows the total number of oat seedlings which were examined in the course of a number of experiments extending over a period of months. The letters refer to the gender of the inoculum used, viz., the first column shows the results with one "A" gender inoculation, the second column refers to double inoculations using "A" gender, the third and fourth columns show these experiments repeated with inoculum of "B" gender, while the fifth column refers to double inoculations using "A" gender followed by "B" gender or *vice versa.*

Table I.—Oat seedlings inoculated with cultures of the same and different genders.

Gender of inoculum.	A.	AA.	B.	BB.	AB.	Control, uninoculated.
Number of seedlings examined	76	24	56	25	204	95
Number of seedlings found uninfected	76	24	56	25	18	95
Number of seedlings found infected	0	0	0	0	186	0
Percentage of infection	0	0	0	0	91	0

It will be seen that in no case out of 180 seedlings examined was any infection obtained with one gender only, but that where two different genders were present, 91 per cent. infection was obtained. The controls remained uninfected throughout. It was found that in any experiment in which infection occurred, the percentage of diseased plants was high, but that there were experiments in which, under apparently similar conditions, no infection was obtained. These aberrant results cannot yet be explained, and are at present a subject of investigation.

Another result of interest was that, provided two genders were present, infection occurred, whether the two genders were of the same species, viz., *Ustilago levis*, or of two different species, viz., *Ustilago levis* and *Ustilago hordei*. It was, however, found that pure oat smut would not infect barley seedlings, and that pure barley smut would not infect oat seedlings. In addition, while at present the cytological evidence is not complete, there is every indication that the hyphæ which infect are "fusion-hyphæ."

I wish to thank Dr. Wm. B. Brierley for the interest he has taken in this work, and for the facilities provided.

Summary.

Experiments are described which show that no infection of oat or barley seedlings by pure cultures of smut fungi occurs when one gender (sex) is present, but when, under similar conditions, two genders are present, 90 per cent. infection and over is obtained.

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Croonian Lecture:—*Organizers in Animal Development.*

By Hans Spemann, Professor of Zoology, Freiburg i. B.

(Lecture delivered November 3, 1927.)

I.—*Introduction.*

One of the most remarkable features of the phenomena of life is their uniformity in the most unlike living forms. This fact enables us to choose for our researches such forms as will be, for one reason or another, the most suitable for observation or experiment. The results thus reached must, however, by no means be generalized without further proof; but again and again observations made on favourable subjects have been confirmed by further study in less favourable ones. The fertilization of the egg, for instance, was observed first in the eggs of the sea-urchin, that is, in a species as unlike as possible to any higher animal; yet it has been shown since that fertilization goes on in the same way in all animals. The beginnings of a human individual do not differ essentially from those of an echinoderm.

I hardly suppose that many of you have up to the present taken any particular interest in the early development of the common newt, my only subject of research for the greater part of my life; I hardly think it is to such interest that I owe the honour of delivering this lecture. I imagine it was rather the conviction that the laws of development established for this low vertebrate hold true for all vertebrates, nay, for all animals; that development even of man follows the same principles. But for the same reason I dare not presume that all of you have present in your mind the first steps of development of the newt's egg. It will, therefore, be my first task to recall them to your memory.

(*a*) *Scheme of Normal Development of the Triton Egg.*—From the fertilized egg, morphologically a single cell, there arises by typical cell-divisions the *blastula*, containing a cavity, the *blastocœl*, formed by cells of very different size, shape and quality. The thick floor of the blastula is composed of the large yolk-laden vegetative cells; its thin roof is formed by the much smaller animal cells, with little yolk; the cells between both of them are middle-sized, forming the so-called marginal zone.

In a predetermined point a little below this zone an invagination takes place, growing deeper and deeper and advancing to both sides; this is the *blastopore*, with the *blastopore-lips*. By this invagination the whole material of the floor and the marginal zone of the blastula is turned inside, forming the *archenteron*.

Its cavity, still communicating with the blastopore, widens in front, whilst at the same time the original cavity of the blastula is reduced to a mere slit. This stage of development is known as the *gastrula*, and the process of invagination by which it is reached is called *gastrulation*.

The cell-layer derived from the animal cells of the blastula is called the outer germ layer or *ectoderm*; it gives origin to the epidermis and the whole nervous system. The floor of the archenteron, the *entoderm*, formed by the large yolk cells of the blastula, is the rudiment of the intestine; in this early stage it is not yet a closed tube, but an open furrow, its sides bending upwards without closing together. The opening of the furrow is covered by a plate of middle-sized cells, called the *mesoderm*. They are derived from the cells of the marginal zone, and form, as it were, the roof of the open archenteron. Out of this mesodermal plate are formed primarily muscles and skeleton, kidneys and peritoneum.

Now, to conclude this description, a few words about the origin of the central nervous system. The ectoderm in front of the blastopore, on the dorsal side of the gastrula, thickens to form the *medullary plate*, broad in front, narrower at the hinder end, surrounded by the *medullary folds*. This plate changes first into a furrow, then into a tube, covered by the surrounding epidermis. It is the *medullary tube*, the rudiment of the brain and spinal cord from the fore-end of this tube the rudiments of the eyes are pushed out, the so-called *primary eyeballs;* a little farther back the *ear-vesicles* are formed, the rudiments of the labyrinth.

(*b*) *Deduction of the Problem of Determination.*—From the facts of normal development thus outlined may be derived the problem with which our experiments were dealing. By an ingenious method worked out by W. Vogt, we may mark a definite part of the young gastrula by staining it *intra vitam* with nile-blue, and may follow it through development. It may thus be subsequently identified, for instance, in the medullary plate and later on in the primary eyeball. This part of the gastrula would then have been *presumptive medullary plate*," "*presumptive primary eyeball*." Another part farther away from the blastopore would prove to be *presumptive epidermis*.

Now the question arises: when will these parts of the gastrula *be determined* to differentiate either to medullary plate or to epidermis? Are these organs perhaps already determined at the beginning of gastrulation? Further development would then be *self-differentiation*, according to Roux. Or will they be determined later on, perhaps during gastrulation?

II.—*Experimental Deduction of the Conception of "Organizer."*

(*a*) *Exchange of Presumptive Medullary Plate and Epidermis.*—This alternative may be decided by a simple experiment. Between two embryos of the same age, young gastrula stage, little pieces of presumptive medullary plate and presumptive epidermis are exchanged. Using embryos of different colour, either natural or stained, we may identify the transplanted pieces for many days and follow them in their development. As the pieces have been exchanged, each of them serves as a mark for the original place of the other piece, showing exactly what it would have become.

In fact, each piece develops not according to its origin, but according to the new place it occupies, presumptive medullary plate becoming epidermis in epidermis, presumptive epidermis becoming medullary plate in medullary plate. Hence it may be concluded :—

(1) That presumptive medullary plate and epidermis are relatively indifferent at the beginning of gastrulation.

(2) That there is some factor acting in the new place that determines the definite fate of the transplanted pieces.

Small pieces of the young embryo may be exchanged as well between different species, *i.e.*, heteroplastically. A very favourable combination is that of *Triton cristatus* and *Triton tæniatus* or *alpestris*, the eggs of the former being almost free from pigment, whilst those of the latter are much darker, especially those of *alpestris*, and densely laden with minute pigment granules.

In this way from a young gastrula of *tæniatus* a piece of presumptive medullary plate was taken, from a gastrula of the same age of *cristatus* a piece of presumptive epidermis ; these pieces were exchanged. Both embryos developed normally. In the *tæniatus* after two days the white implanted piece of *cristatus* was seen in the medullary plate, fore end left side. Later on, when the folds had closed and the brain with the eyeballs was formed, the light piece was still visible shining through the skin. On sections through the head it is found in the brain and primary eyeball of the left side forming part of their wall. It is somewhat thicker than its surroundings, its cells are larger and free from pigment. It had been presumptive epidermis of *cristatus* and has formed a piece of brain, but brain of *cristatus*, amidst the brain of *tæniatus*. The embryo of *cristatus*, from which this piece had been taken, showed the implanted piece of *tæniatus* on the right side in the skin, in the region of the gill rudiments. Though presumptive brain, it had become epidermis, amidst the epidermis of the host ; but epidermis of *tæniatus* according to its origin, with all its tendencies of growth. This could be seen by the form of the gills, which was that of *tæniatus.* The gills were further developed on the operated side; they contained rudiments of blood vessels of which nothing was to be seen on the other side.

(*b*) *Ectoderm and Mesoderm exchanged.*—By the same method O. Mangold examined the potencies of ectoderm and mesoderm, exchanging pieces of them at the beginning of gastrulation. He found that a piece of presumptive ectoderm transplanted in the region of the blastopore may be invaginated with the surrounding material and give rise to mesodermal organs such as notochord, somites, kidneys.

(*c*) *The Centre of Organization.*—Not all parts of the embryo are as plastic as this. A piece taken from the upper lip of the blastopore and transplanted into an indifferent region of another gastrula behaves in a totally different way. It does not adapt itself to the development of its surroundings, but sticks to its own way and forces the surrounding parts to follow its own direction. There arises a second embryo composed partly of the transplanted material, partly of cells of the host that have been induced to adapt themselves to the development of those ruling cells.

This experiment was made for the first time on my suggestion by Hilde Mangold. In the most instructive case which she obtained, a secondary embryo was formed with a medullary tube bearing ear-vesicles on its fore end, with notochord and two rows of somites. It was composed of cells of *cristatus* derived from the implanted piece, and cells of *tæniatus*, the host, that had adapted themselves to their development. The boundaries of the implanted piece, though very sharp and clear, were not respected by the general form.

Such a transplanted piece "organizes" its new surroundings; I therefore call it an "organizer." It induces a "field of organization." The region of the early gastrula where these organizers lie may be called for the present a "centre of organization."

III.—*Experimental Analysis of the Function of the Organizer.*

(*a*) *Extent of the Centre of Organization.*—This point being reached we proceeded to a methodical analysis of the organizing faculty. The next question presenting itself was that of the extent of the centre of organization. After some preliminary experiments by Otto and Hilde Mangold, this question has been definitely settled by H. Bautzmann. He examined the surroundings of the beginning blastopore by transplanting small trial-pieces into the blastocœl of another gastrula. By gastrulation these pieces are brought under the ectoderm and may show their power of induction. The region of the gastrula so determined as "centre of organization" occupies a semicircular area above and beside the upper lip of the blastopore. This area coincides with that part of the gastrula which had been shown by W. Vogt to invaginate during gastrulation and form notochord and mesoderm.

This result is in harmony with a statement made by another student of mine, A. Marx, that a medullary plate may be induced by a piece of the roof of the archenteron, that is, of the material for notochord and mesoderm. The experiments of Bautzmann show, in addition, that these cells have this power even before gastrulation, and may not only induce medullary plate in the overlying ectoderm, but also mesodermal organs in their new environment.

(*b*) *Origin of the Centre of Organization.*—In the young gastrula the centre of organization is already determined; when does it originate? Probably very early in development, together with that shifting of material of the unsegmented egg that follows fertilization and produces the well-known "grey crescent," that area of the egg where later on invagination begins. The facts which point in this direction are the following: If in the 2-cell stage the dorsal blastomere is eliminated, that is, that part of the egg which contains the grey crescent, the ventral blastomere, though going on developing, and even gastrulating, forms no axial organs, that is, no medullary plate, notochord, somites. The same holds true for the case in which the dorsal half of the *unsegmented egg* has been cut off.

An older experiment of M. Moszkowski teaches the same thing still more conclusively. In the unsegmented egg the grey crescent was destroyed by puncture with a hot needle; the egg developed; it even gastrulated, but formed no axial organs. The crucial evidence would be induction of a medullary plate by a transplanted piece of the grey crescent. Such a piece when containing a supernumerary spermatozoon develops in the blastocœl. This experiment has been performed by me many times, but has not yet yielded a clear and positive result.

(*c*) *Intimate Structure of the Centre of Organization.*—Several facts are known concerning the intimate structure of the centre of organization.

Longitudinal Structure.—It is a significant fact that the secondary embryo need not have the same orientation as the primary. If it had, this would have pointed to some intimate structure of the host, in accord, perhaps, with Child's idea of gradients. Now such gradients may exist in the host and may be effective; there are reasons to believe it. Yet their influence is surely not decisive. The induced secondary embryo may even lie at right angles to the primary one. This is most striking when ear-vesicles are formed; they lie symmetrically to the right and left of the secondary medullary tube to which they are attached, but in relation to the host one behind the other. Therefore the organizer must have some longitudinal structure, which is not lost by transplantation and which determines the direction of its invagination and consequently of the induction. To clear up this question, organizers of oblong shape should be implanted in definite directions. Such experiments have been begun by Dr. Geinitz some years ago and have now been taken up by myself.

Laterality.—There must also be some medio-lateral structure of the centre, "laterality," to use Streeter's term. This may be concluded from experiments carried out quite recently by W. Vogt and K. Görttler, the knowledge of which I owe to the kindness of the authors. W. Vogt experimenting on Triton retarded the development in limited parts of the egg, either by localized lack of oxygen or localized cooling. For the latter purpose a piece of thin silver-plate was perforated by a hole, just large enough to hold a salamander's egg, deprived of its shell. The plate was fixed in a chamber, dividing it in two parts, in which water of different temperatures was circulating. The communication between the two parts was then closed by a salamander's egg, as by a cork. In consequence the two halves of the egg developed at very different rate; an embryo was produced that was composed of two halves of very different "age," with a perfectly sharp limit between them. For example, in one case, where the limit was transverse, there resulted an embryo with a rather long tail, but instead of a brain it had only a medullary plate.

Of greater interest for the question we are dealing with are such cases where the limit is median, dividing two lateral halves of very different age. From this Vogt concludes that the two halves developed independently from each other, as Roux had concluded from his classical experiments on half embryos. Vogt further concludes that this development is based on laterality of the two halves already existent in the 2-cell stage.

By a strange coincidence Prof. Julian Huxley had been performing similar experiments at about the same time, which have just been published in the last few days in the jubilee volume for Hans Driesch. His method differed from that of Vogt in that the gradients of temperature he applied were much less abrupt, so that his embryos did not show such a sharp limit between the two halves.

K. Görttler cut out a lateral piece of the upper lip of the blastopore and replaced it by the corresponding piece of the other side of another embryo, so that the gastrula went on developing with two left lips. Then two left halves of the medullary plate were formed, with two left medullary folds. The left half of the lip, as we must conclude, had some intimate structure that made it " left " ; that is, it had laterality.

This experiment of Görttler reaches farther than that of Vogt. From the results of Vogt a half-structure could only be inferred for the embryo as a whole, whilst the results of Görttler prove a half-structure more especially for the centre of organization, that is, of the presumptive mesoderm, which is invaginated during gastrulation and induces the medullary plate. The ectoderm of which the latter is formed, the presumptive medullary plate, may have had a similar structure ; but this very fact would show the dominance of the organizing cells which can reverse the laterality of the induced material.

Regional Structure.—There are some facts that indicate yet another kind of structure of the centre of organization. This might be called a *regional structure*, as it determines the regions both of the mesoderm and of the medullary plate induced by it.

In the first perfect case of induction described by Hilde Mangold the ear-vesicles lay at the tip of the medullary tube ; which means that the greater part of the brain with the eyeballs was lacking. Now I produced an isolated brain, with well-developed eyeballs in front and two ear-vesicles at the tip of the free posterior end. These different results may be caused by a difference in the inducing organizers, which might have been taken from different regions of the organizing centre. That mesodermal material which would come to lie under the anterior part of the medullary plate, would induce the brain ; the material underlying the posterior part of the plate would induce the spinal cord.

In order to test this a piece of the upper lip may be excised at different stages of gastrulation and transplanted to the same region of another gastrula. As the mesoderm is wandering round the upper lip, the material passing it first will be brought to the anterior end and lie under the brain, whilst the material invaginated later will occupy the more posterior parts of the embryo lying under the spinal cord.

This experiment has been performed by me this summer. It proved that the centre of organization really has some regional structure, but at the same time it showed that the question is more complicated. The organizer may induce more than it ought to do ; it has the tendency to build up a whole, behaving in this like a harmonious equipotential system of Driesch. And, further, it seems to make a difference according to the region of the host into which the organizer is implanted. Therefore the experiment must be varied still in another sense; as *different organizers* have been transplanted to the *same place*, so the *same organizers* must be transplanted to *different places.* I have made this experiment, too, last summer; it yielded the result just stated.

(*d*) *Nature of the Inducing Agent.*—Of still greater interest than extent, origin and structure of the organizing centre, is the nature of its action; but obviously here our knowledge is very scanty. On the one hand, it may be said that this action cannot be of quite indifferent nature, *e.g.*, contact-action; for a piece of presumptive epidermis does not act in the same way. The stimulus must be *specific* to a certain degree. On the other hand, the stimulus is clearly not specific *within narrow limits.* This is shown by experiments of B. Geinitz. He found that induction may take place between an organizer of a frog, or toad, and a newt; that is, not only between embryos of different species or genus, but even of different order.

So far we know hardly anything about the most elementary question, whether induction is performed by chemical or by dynamical means. If the latter were true, only the living organizer could be supposed to induce; whilst morphogenetic substances might remain effective even after destruction of the cells. This shows the way this problem may be attacked.

In this connection I wish to mention two facts of a very promising character stated quite recently. One of them has been found quite independently by O. Mangold and myself in 1926; it might be called "*homoiogenetic induction*" or "*assimilating induction.*" We transplanted a piece of medullary plate into the blastocœl of a gastrula in order to bring it under the epidermis from the very first. In this way we made the surprising discovery that medullary plate may induce its like, that is, medullary plate. Mangold transplanted heteroplastically; I did so with stained material. We followed the whole development in the living object and on sections of preserved material, so we are quite sure of the fact itself. To explain it, we may suppose that the inducing agent may be given off again by the medullary plate which it had formed, to induce in indifferent material a new medullary plate.

Last summer, Mangold made the further discovery, not less important, that this agent is retained in the medullary substance for a surprisingly long time. A piece of the brain of a free-swimming larva may still induce a medullary plate. This fact seems to involve the chemical nature of the inducing stimulus. The case is similar to another known for many years, the regeneration of the lens of Triton from the upper margin of the iris, as discovered by Colucci and G. Wolff. According to the experiments of H. Wachs the lens regenerates under an inducing influence of the retina, the same part of the eye that induces the lens during the normal development. It must have retained this faculty which was needed during only a short time.

This seems to be of more general occurrence. Last summer, H. Bautzmann found something very similar. A piece of the very young notochord cut out of a neurula and transplanted into the blastocœl induced in the ectoderm a medullary plate. The notochord must have retained this faculty which had become active a long time before, when inducing the original medullary plate of the neurula.

IV.—*The Rôle of the Organizing Faculty in Normal Development.*

What rôle does this organizing faculty play in normal development? It has been shown by numerous experiments that the rudiment of the eye, the eyeball, may induce the formation of a lens in strange epidermis. It is very probable, therefore, that the eyeball exerts the same influence in normal development. The eyeball may therefore be called the organizer of the lens. Now an eyeball may be produced experimentally from such material as in normal development would have become epidermis, material that was transplanted to its new place and had thus been induced to form an eyeball. It is again very probable that in normal development the material of the eyeball is determined in the same way, by an inducing influence of the underlying mesoderm. Induced itself, it goes on inducing; so it may be called an *organizer of second grade.*

Another organizer, of second grade, B. Geinitz and I have produced by the following experiment. As I said before, O. Mangold has produced mesoderm out of presumptive ectoderm by transplanting presumptive epidermis of a young gastrula among presumptive mesoderm of another gastrula. This transplanted piece took part in the invagination and the further development of its new surroundings, where it formed notochord, somites, kidneys. B. Geinitz and I repeated this experiment with the same effect. Now Marx had shown, as I said before, that mesoderm may induce medullary plate. This experiment, too, has been repeated many times with the same result.

The question arises whether this inducing faculty of the mesoderm is acquired even by a piece of ectoderm that has been turned (experimentally) into mesoderm. To test this we combined the two experiments; that is, we forced presumptive ectoderm by transplantation to become mesoderm, took it out again some time after invagination and transplanted it afresh into the blastocœl of a young gastrula. In this way it was brought under the epidermis, where, in fact, it induced a highly perfect medullary plate.

In this experiment, as in the former, two processes of development, each of which had been determined by induction, were linked together. It seems to be not improbable that the whole development, at least of the amphibian embryo, is composed of single processes connected by organizers. The special mode of combination would have to be stated in the separate concrete cases; but in principle the old problem would be solved strictly according to the theory of epigenesis.

But as a matter of fact matters are much more complicated. About this let me add a few words in conclusion.

The experience gained by studying the development of the lens should make us cautious. There, as we have seen, the eyeball may induce a lens in foreign epidermis; but other experiments showed, with not less evidence, that the normal lens cells may also develop independently, forming a lens without an eyeball. In one subject at least (*Rana esculenta*, the water frog), both faculties have been shown conclusively to coexist, though either by itself would seem sufficient to warrant normal development. Rhumbler named this what engineers call "double assurance," and Braus adopted this term for a very clear similar case he had discovered.

The first rudiments of organs might be determined in the same way; that is, the ectodermal material which is going to form the medullary plate might come to meet the inducing action which the underlying mesoderm exerts; this might even go so far that the material would form a plate without mesoderm. K. Görttler, examining this question during the last few years, has recently attained a clear positive result. I will only quote his last experiment which I consider to be the most conclusive and important. Presumptive medullary plate of a young gastrula was transplanted into the epidermis of a neurula; that is, of a somewhat older stage. There it developed sometimes in accordance with its new place, that is, to epidermis instead of medullary plate, as I had always observed after transplantation into a host of the same age; but sometimes it formed medullary plate in accordance with its origin.

This difference in behaviour Görttler believes to be caused by different orientation of the implanted piece. It develops according to its original destiny, if the intended movements of cells be not hindered by the new environment. There is a shifting of cells connected with development both in the implanted piece and in its new surroundings. If they are in harmony, the piece will follow its original intention and form medullary plate; if both interfere, it gets disturbed, as it were, and will follow the development of its new surroundings. This would show that even at the beginning of gastrulation a certain region is determined, though not irrevocably, to form medullary plate; mesoderm is then invaginated and pushed under it and acts in the same sense. How this harmony arises, that is the great question, and new experiments alone can answer it.

V.—*Summary.*

To summarize: The conception of organizers in development has been derived from experiments on amphibian embryos in the earliest stages. The different regions of such an embryo have not the same value for development; most of them are relatively indifferent and do not carry their destiny wholly within themselves. This can be shown by transplantation of these parts into other regions of the embryo; they may follow the development of their new surroundings. But there is a certain region in the embryo, parts of which behave in an entirely different way. Transplanted into a more indifferent region of the embryo they do not adapt themselves to their new surroundings, but retain their own character, and force the others to follow them. Such pieces "organize" a new embryo, which is built up partly by the transplanted cells, partly by the cells of the host. For that reason they were called "organizers," while the region where they lie in those early stages of development was called the "centre of organization."

Further experiments were directed to determine the extent of this centre, its origin, its intimate structure and the nature of the organizing influence. The organizing centre coincides with the presumptive mesoderm; transplanted pieces taken from this region not only induce medullary plate, but also determine their new mesodermal neighbourhood to form notochord and somites. This centre seems to be foreshadowed by the grey crescent; defect of grey crescent prevents formation of axial organs. The cells of the centre must have some longitudinal structure which determines, at least partly, the direction of invagination and the orientation of the secondary embryo. There may be a corresponding structure in the ectoderm, but it is less effective. The same holds true for a regional structure which determines the regions of the medulla. All these processes obey the principle of "double assurance." The means of induction seem to be rather material than dynamical.

What has been achieved is but the first step; we still stand in the presence of riddles, but not without hope of solving them. And riddles with the hope of solution—what more can a man of science desire?

The Enzymatic Function of Mitochondria in the Germination of Cereals.

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(Communicated by Prof. A. J. Ewart, F.R.S.—Received July 11, 1927.)

[Plates 7–12.]

CONTENTS.

	Page
Introduction	188
Methods of Investigation	191
The Metabolism of Germinating Maize	191
The Behaviour of Mitochondria in the Scutellum and Endosperm	193
The Relation of Mitochondria to Enzymatic Activity	196
The Behaviour of Mitochondria in the Aleurone Layer	197
Auto-Digestion in the Endosperm	198
Secretion of Mitochondria from the Scutellum in Germinating Wheat and Barley	200
Concluding Remarks	200
Summary	201
References	202
Description of Plates	204

Introduction.

The significance of mitochondria in plant cells is a subject to which a great deal of attention has been paid in past years, and from the studies of many cytologists it has become apparent that these bodies are fundamentally concerned in the formation of many different substances in the cell. Fat droplets, anthocyanins, essential oils (28) and protein grains may all be quoted as examples of such substances (Cowdry, 5). Starch and other plastid products also appear to owe their origin indirectly to mitochondria ; for it has been shown by various observers that plastids are in reality enlarged and transformed mitochondria, which take on the varying functions of the production of starch, chlorophyll, anthocyanins, fat, etc., according to whether they become amyloplasts, chloroplasts, chromoplasts, elaioplasts or other such protoplasmic structures. It seems, moreover, that these substances are originally formed within the mitochondria, which later enlarge to form the body of the plastids (Cowdry, 6).

A suggested explanation of this productive activity of mitochondria is found in the eclectosome theory of Regaud (32), according to which mitochondria play the rôle of plasts, selecting materials from the cytoplasm, and fabricating them in their interior into various products. More in accord with known physicochemical processes, however, is the now generally accepted interpretation recently expressed by Cowdry (7), wherein the phase-boundary of the mitochondria and the surrounding cytoplasm is regarded as the seat of processes of elaboration, beginning with adsorption of the molecules of certain solutes, and ending with a series of chemical or physical interactions between the mitochondrial material and the incoming substances--such interactions leading to the building up of new compounds of widely different character.

Further light is thrown upon the mechanism of this synthetic activity by the recent development of an enzymatic concept of mitochondrial behaviour, a development mainly due to the writings of Marston and of Robertson in the University of Adelaide. Marston (25) concludes on the basis of their reactions to azine dyes that mitochondria contain proteolytic enzymes ; and, considering the rapidity with which synthetic processes take place in living tissues (26), he develops the conception of mitochondria as the site of enzymatic syntheses in the cell, the " water-poor " phases which exist at the surface of the lipoidal constituents of the mitochondria providing suitable conditions for synthetic activity.

An interesting suggestion has been added to this conception by Robertson (34), who points out that the lipoid constituents of the mitochondria would cause contained molecules to be orientated at the surface in such a manner that the reactive groups (for example, the carboxyl and α-amino groups of amino-acids) would point towards the external aqueous phase, and so be effectively concentrated at the phase-boundary of mitochondrium and cytoplasm. In the light of de Nouy and Cowdry's conclusion that the mitochondrial substance is arranged in such a way as to give a surface film of maximum extent with minimum amount of material (27), such a local concentration would furnish an explanation of the facility with which enzymatic syntheses take place at the surface of these bodies in the cell. Marston's assertion that proteolytic enzymes are concentrated within the mitochondria makes it appear that herein lie practically ideal conditions for the synthesis of proteins. Marston has, moreover, added considerable support to this conclusion by showing *in vitro* that the presence of colloidal lipoid emulsions greatly increases the reaction velocity of the synthesis of protein from its tryptic digestion products. The evidence is therefore strong in favour of the enzymatic synthesis of proteins taking place at the mitochondria-cytoplasmic interface.

And, if this is so in the case of proteins, might not enzymes, situated in the mitochondria and acting at their surface, perhaps be concerned in, and explain the synthesis of, many other substances which, as we have seen, appear to owe their origin to these cell-bodies? The synthesis of starch, the synthesis of fat, etc., might conceivably be the result of amylase, lipase, and other enzymes being contained in mitochondria or the derived plastids; and the confirmation of such a suggestion would complete the conception of the mitochondria as the seat of all constructive activity in the cell.

It is not, however, in synthesis only that enzymes are agents in cell-metabolism: processes of hydrolysis and digestion are also activated by enzymes, and it is a potent fact that mitochondria have been shown to be concerned in these cases likewise. For Regaud and Mawas (33) believe that the zymogen granules which are secreted from the salivary glands, and are regarded as a kind of resting state of the enzyme, arise from mitochondria; while in the case of the cells of the pancreas it has been observed that the mitochondria break down to form the zymogen granules which are passed into the acini, thus indicating that the mitochondria are the source of the tryptic secretion (Arnold, 1; Horning, 16). In the process of intracellular digestion in *Paramœcium* and in *Nyctotherus* it has been shown that mitochondria come to be included in the food vacuoles, suggesting that subsequently the mitochondria give rise to the enzymes concerned in the digestive activity (Horning, 17, 19). Observations have been made also on the relation of mitochondria to the process of intracellular digestion in living amœbæ (Horning, 18). The engulfed food at first circulates in the protoplasm, at the same time coming into contact with mitochondria, which then adhere to it. Subsequently a vacuole forms round the food particle with its associated mitochondria, after which the latter pass into solution and disappear, and the food undergoes disintegration. It is thus again concluded that the mitochondria are concerned with the production of the enzymes which bring about the digestion of the engulfed food materials.

Without entering upon a consideration of the factors governing the reversal of the direction of enzyme-controlled reactions, it appears then that under some conditions the mitochondria are concerned in synthesis with enzymes active at their surface, and that under others they migrate to certain regions and liberate the enzymes which then effect processes of hydrolytic degradation. There is, however, pressing need for further inquiry into this enzymatic behaviour of mitochondria, and especially into their migration subsequent to enzymatic secretion. In particular it has seemed highly desirable to institute an inquiry into the activity of mitochondria during enzymatic secretion in plants, an activity about which nothing whatever is known. For this purpose, therefore, an examination was made of the behaviour of these bodies during the germination of the caryopsis of certain cereals, the results of which form the subject of the present communication.

We desire to express our appreciation of the helpful criticism and suggestions we have received during the pursuit of this inquiry from Prof. A. J. Ewart, F.R.S., Prof. W. E. Agar, F.R.S., and Dr. O. W. Tiegs.

Methods of Investigation.

In order to carry out this investigation cereal grains were germinated at a temperature of 25° C. in damp sawdust for the earlier stages, and in moist soil when older material was required. The types used were Red Hogan maize, Warden wheat, and Cape barley.

In the study of this material great success was obtained by the use of hand-sections stained *intra vitam* with Janus green B (1 : 8000). This dye rendered the mitochondria clearly visible without staining any of the other protoplasmic constituents. Observations made from preparations of this nature were, however, amply supplemented by the use of material fixed in an osmo-chromic fluid, in Champy-Kull solution, or in Cox's fixative. After fixation such material was washed in running water for 24 hours, embedded and sectioned, the sections being stained in iron hæmatoxylin and sometimes counterstained in eosin. Examination of these preparations with high magnification revealed the mitochondria as dark bluish granules in the protoplasm.

After fixation in absolute alcohol these granules were not rendered visible by staining in iron hæmatoxylin; and this, added to the observations made upon permanent mitochondrial preparations and the sections stained with Janus green, led us to believe that the granules studied were of the same nature as the mitochondria found in the tissues of other plants and animals.

The Metabolism of Germinating Maize.

The changes effected in the maize caryopsis during germination have been studied on numerous occasions from the pioneering investigations of Sachs (35) and Detmer (9) to those more recently carried out by Reed (31) and Toole (39). In the resting state of the grain the endosperm cells are densely packed with starch grains, except for several layers immediately adjacent to the scutellum which, judging by analogy with the observations of Brown and Morris on barley (3), are to be regarded as having been emptied and having had their thin walls crushed together in the pre-resting stage. In the resting state also the epithelial cells of the scutellum contain very finely granulated and semi-transparent protoplasm; shortly after the onset of germination, however, the protoplasm of the epithelial cells becomes much coarser in structure and the granulations increase greatly in size and number—changes to be associated with the assumption of secretory activity (Reed). Then, after about 24 to 36 hours, corrosion of starch grains commences in the endosperm cells adjacent to the scutellum, and sugars and proteins appear there in solution.

These hydrolysed materials are absorbed by the scutellar epithelium, and pass on to the embryo to be utilised in the process concerned in its growth. As germination proceeds, the depletion effected in this manner extends slowly to the farther portions of the endosperm: simultaneously the scutellum, probably as a result of colloidal hydration associated with the general swelling of the awakening seed, undergoes considerable increase in size, which results in further crushing of the depleted portion of the endosperm, and in the epithelium being thrown into glandular folds (Sargant and Robertson, 36). At the same time the epithelial cells themselves become elongated and often swollen at the ends, and lose their lateral adhesion to one another; they may even form hypha-like protuberances into the endosperm which are often of considerable length. Finally, when the endosperm is entirely depleted, the protoplasm of these cells loses its granular appearance and becomes perfectly clear.

There has been a certain amount of controversy upon the tissues involved in the elaboration of the enzymes concerned in the hydrolytic processes during germination. Diastase is present in the grains of cereals even in the resting stage (Kjeldahl, 20; Brown and Morris, 3); but there is still a little doubt whether it is secreted by the scutellum or arises entirely in the endosperm. And of the proteolytic enzymes which White (41) has shown to occur in maize, we have no indication whatever of the place of origin.

The cytological researches of Torrey (38), Reed (31) and Sargant and Robertson (36), however, strongly suggest that the amylo-hydrolytic enzyme is formed in the embryo. They succeeded also in localising the origin of the enzyme in the epithelial layer. These results have more recently been confirmed by Mann and Harlan (24). Similar conclusions to those of Brown and Morris were reached by van Tieghem (40) in the case of the excised embryos of various other plants.

Bruschi (4), however, criticises the results of these investigations on the ground that bacteria may have been present in the cultures, and that these may have hydrolysed the starch and rendered it available for absorption by the embryos; and Linz (22), although he detected diastase in the scutellum by quantitative estimation, states that isolated embryos do not liberate the slightest trace of the enzyme, a conclusion also reached by Grüss (12).

Nevertheless, the more reliable evidence seems to indicate that there is a definite enzymatic secretion from the scutellar epithelium into the endosperm; and we are inclined to agree with the recent opinion of Toole who, after a careful study of the germination of maize, concludes that, although (as will be described later) it is possible for sugars to appear in the endosperm without any evidence of activity of the epithelial cells, there is nevertheless strong evidence in favour of the epithelial layer being the main source of enzymes for the digestion of the materials composing the endosperm.

The Behaviour of Mitochondria in the Scutellum and Endosperm.

Examination of the resting stage of the grain showed the endosperm densely packed with small round starch grains, excepting the crushed layer of depleted cells adjacent to the scutellum. The epithelial cells of the latter organ were small and compact, with densely staining protoplasm which, in material fixed in osmo-chromic fluid, was seen to contain numerous very small brown-stained lipoidal droplets. The nuclei of the epithelial cells, as Reed has described, contained only a small amount of chromatin, which occurred in spheres round the inner surface of the nuclear membrane. The remaining cells of the scutellum were highly vacuolated and also contained large numbers of lipoidal droplets of varying size. Mitochondria were to be observed in moderate frequency both in the epithelial and the other cells of the scutellum; they also occurred sparsely among the starch grains in the cells of the endosperm. These features of the resting stage are illustrated in Plate 7, fig. 1.

Examination of material from three to six days after the commencement of germination showed the beginning of depletion of the endosperm cells immediately adjacent to the scutellum; some of these cells were completely empty and in others starch grains were showing the typical appearance of being in process of corrosion. The scutellum and its epithelial cells had also increased considerably in size, causing further crushing of the depleted cells. What was of most striking significance, however, was the profusion of mitochondria now to be seen in the scutellum and its epithelial cells. The parenchyma cells of the scutellum were still seen in osmo-chromic preparations to be densely filled with lipoidal droplets, the smaller of these clustering round the vacuolar membranes, the larger ones often occurring within or entirely occupying the vacuole; the narrow strands of cytoplasm separating these droplets, however, contained numerous mitochondria (Plate 8, fig. 2), clearly shown in the Cox's and Janus green preparations, and in sections of material fixed in osmo-chromic fluid differentiated from the brownish-black lipoidal droplets by their bluish staining reaction with hæmatoxylin. Mitochondria were also sometimes seen in masses in the intercellular spaces. They were equally abundant in the epithelial cells, often aggregated profusely at their distal ends; Janus green preparations frequently showed them thus in masses so dense and deeply stained as to have lost all detail (Plate 10, fig. 4). Of the greatest significance, however, was the fact that mitochondria were clustered in numbers round the outer wall of many epithelial cells, often appearing partly embedded in the cell-wall (Plate 8, fig. 2).

These features strongly suggested that the mitochondria had actually passed from the interior of the epithelial cells through the walls into the endosperm. Large numbers of these bodies, often closely packed, were observed in the space between the epithelium and the crushed boundary of the endosperm; and, while on account of the thinness of paraffin sections this space sometimes appeared empty, in the thicker hand-sections stained with Janus green B it was found always to contain mitochondria in greater abundance than in any other part of the material. Numerous scattered mitochondria were also found embedded in the crushed layer of endosperm cell-walls itself (Plates 9 and 10, figs. 3, 4). In these respects the germinating grain differed markedly from that in the resting state, wherein the space between the epithelium and endosperm was perfectly clear and devoid of contents, and no granules were visible on the exterior of the epithelial cells (*cf.* figs. 1 and 2). This apparent process of secretion commencing with the onset of germination continued throughout the period of endosperm depletion.

It is to be supposed that the mitochondria arise in the epithelium by repeated division; their peculiar sensitivity generally renders this difficult of observation (see (6), p. 329), although occasionally they were observed in the elongated form which they assume prior to fission. In later stages the external aggregation of mitochondria was sometimes very dense even in 3 μ sections, in which case the secreting cells appeared comparatively clear and devoid of granules, suggesting that their secretory activity had at all events temporarily ceased. To what extent the mitochondria are identical with the granulations observed in the epithelial cells by Reed (31) it is impossible to say; both the granules of Reed, however, and the mitochondria of our own preparations disappeared on cessation of secretory activity.

In the early stages of germination, *e.g.*, about six days after its inception, the abundance of mitochondria outside the epithelial layer was observed also to extend into the adjacent endosperm cells which were undergoing depletion. Large numbers of mitochondria were clustered along the walls of these cells, some appearing even to be partly or wholly embedded in the walls themselves ; others, either singly or in masses, occurred scattered in the now emptying lumina ; and, while the starch grains in an advanced stage of corrosion were generally free, mitochondria were in many cases seen aggregated in numbers round those grains which had not appreciably begun to dissolve, sometimes indeed to the extent of completely enshrouding them. These features are illustrated in figs. 3 and 4.

At the same time in the endosperm cells farther from the embryo, wherein no depletion had yet occurred, the number of mitochondria was distinctly greater than in the resting stage : these bodies now occurred in little clumps in the interstices of the closely packed starch grains, and also promiscuously along the walls of the cells (fig. 4). It must be made quite clear, however, that, although the mitochondria were more numerous in these cells now than in the resting stage, their number was still small in comparison to the profusion occurring near the scutellar epithelium and in the cells undergoing depletion.

As germination proceeded, the layer of depleted endosperm cells was always seen to contain a multiplicity of mitochondria, which extended as far as those cells in which starch was actually in process of hydrolysis. These mitochondria were for the most part found in contact with or adjacent to the cell-walls ; but it is conceivable that some originally present in the centre might have become lost during the preparation of the material.

The zone of depleted endosperm cells does not broaden as germination proceeds, since the scutellum continues to increase in size and crush together the walls of the empty cells. Thus towards the close of endosperm depletion the layer of crushed cells was very thick and contained numbers of mitochondria, many of which were undeniably within the cell-wall substance itself. At the close of endosperm depletion, too, the epithelial cells became comparatively devoid of mitochondria and the presence of secretion could no longer be observed (Plate 11, fig. 5).

From the evidence of these observations, which were repeated with great care on a considerable amount of material, we were able to establish beyond all doubt that there is a large increase in the number of mitochondria in the cells undergoing depletion adjacent to the scutellum, and that these mitochondria are associated intimately with the hydrolysis of the starch grains.

Some of the mitochondria must be considered to have originated *in situ*, since, as we have observed, those present in the resting state are able to undergo multiplication. In addition to this, however, the supply is continually supplemented by secretion of mitochondria from the scutellum; and, numerous mitochondria being observed in the layer of crushed cell-walls and in the substance of the walls of the cells still in process of depletion, it may be concluded that those secreted from the scutellar epithelium are able to migrate into the depleting cells of the endosperm in considerable numbers. It is thus not inconceivable that the scutellum may constitute the source of the majority of the mitochondria concerned in starch hydrolysis.

The Relation of Mitochondria to Enzymatic Activity.

We have already pointed out that during germination a secretion of diastase is generally held to take place from the epithelial layer of the scutellum into the adjacent endosperm cells, and that it is this secreted enzyme which is mainly responsible for the hydrolysis of the starch reserve. It was also manifested that a certain amount of evidence has accumulated to show that enzymes are in some way intimately associated with mitochondria: not only has it been shown that lipoidal droplets, such as mitochondria appear to be, accelerate at all events the synthetic activity of enzymes, but mitochondria are believed to contain the enzymes within them; and in hydrolytic processes it has been shown in several instances that the enzymes apparently pass to their seat of activity in actual association with mitochondria, which then dissolve or disappear and liberate the enzyme in an active state.

In the light of these considerations, the observed behaviour of mitochondria during germination of the maize caryopsis becomes fraught with a profound significance: it is indeed remarkable that, bearing in mind the new enzymatic concept of mitochondria, and knowing that diastase is secreted from the scutellum, we should find on observation that a secretion and migration of mitochondria occurs also. Such a discovery suggests that the diastatic enzyme must be secreted from the epithelial cells of the scutellum in association with mitochondria; and in the light of this interpretation the migration of these bodies and their aggregation round the starch grains at once becomes intelligible. This aggregation phenomenon was always observed in the case of intact or slightly corroded starch grains in the cells undergoing depletion; and it seems to suggest, just as did the similar aggregation round the food granules in *Amœba*, that under these circumstances the associated enzyme is liberated prior to commencing its hydrolytic activity. Since it has been shown that the mitochondrial surface is one where the reverse processes to hydrolysis are favoured, such a liberation from the mitochondria of the enzyme would perhaps be expected.

Upon the subsequent fate of the mitochondria we have no direct observations; but the freedom from mitochondria of the starch grains in advanced stages of corrosion, and the absence of any indication of increase in the quantity of these bodies as germination proceeded, suggests that they underwent solution after release of the enzyme, as they do in the food vacuoles of *Paramœcium* (17) and *Amœba* (18). One is led to suspect some change in the condition of the medium leading to release of the enzyme and also perhaps some kind of simultaneous hydrolysis of the components of the mitochondria leading to their solution in the aqueous phase.

The Behaviour of Mitochondria in the Aleurone Layer.

While investigating these phenomena connected with enzyme secretion from the embryo, there were certain other processes in the metabolism of the germinating grain in connection with which it was thought the behaviour of mitochondria might be of interest. Thus a number of authors have adduced evidence for believing that there is a secretion of enzymes from the aleurone layer also.

This conclusion was reached originally by Haberlandt (14), who maintained, indeed, that the whole of the diastase concerned in starch hydrolysis in the endosperm of grasses comes from these cells. His evidence lay in the demonstration of the solution of starch grains which are placed on the isolated and washed aleurone layer. In a critical consideration of his work, however, Brown and Morris (3) conclude that Haberlandt's interpretation of his results is unjustified; and they showed that depletion in barley was no more marked adjacent to the aleurone layer than in the rest of the endosperm. Brown and Escombe (2), however, in reinvestigating this work, admitted that the aleurone cells of barley have both a cyto-hydrolytic and an amylo-hydrolytic capacity; while more recently Grüss (13) claims to have demonstrated the occurrence of corrosion in the endosperm cells immediately adjacent to the aleurone layer. This he did in grains from which the embryo had been removed and which had been placed for four weeks on glass wool saturated with water. Grüss concludes also that oxidising enzymes are secreted from the aleurone layer in the case of barley, a possibility entertained likewise by Stoward (37).

While there is some doubt as to the reliability of the tests used by Grüss for diastase, it seemed a matter of interest to know whether there was any secretion of mitochondria from the cells of the aleurone layer into the starchy endosperm.

As has generally been found by other investigators, even in the later stages of germination there was little indication of withdrawal of the protein and lipoid reserves of the aleurone cells; and in all the material examined the cells next to the aleurone layer were invariably the last to be depleted, and the mitochondria associated with this process showed no evidence of coming from the aleurone layer. The aleurone cells contained a moderate number of mitochondria, but we were unable at any time to obtain evidence that these migrated through the thick walls into the adjacent endosperm cells; and, while the number of mitochondria increased in the latter cells from that characterising the resting stage, this increase was only collateral with that occurring throughout the endosperm, to which reference has already been made (fig. 6).

In addition to these observations, the embryo was dissected out of a number of grains which were then mounted on plaster-of-Paris pillars immersed in water according to the technique adopted by Hansteen (15) and Puriewitsch (30). These experiments were carried out under strictly aseptic conditions, the grains being previously sterilised by the calcium hypochlorite method of Wilson (42). The aleurone layer was examined in grains treated in this manner at intervals up to a month after commencement of the experiment, but with no different results to those obtained in the case of normally germinating grains.

The behaviour of mitochondria, then, lends no support to the view that the aleurone cells secrete enzymes into the rest of the endosperm. One might suggest that the densely protoplasmic condition of these cells, which has influenced various authors (*e.g.*, Haberlandt, 14) in their belief that these cells are endowed with secretory power, would find complete interpretation in the light of their cambial activity during the development of the endosperm within the embryo-sac in the pre-resting stage of the grain (Gordon, 10).

Auto-Digestion in the Endosperm.

It has been shown on numerous occasions that the starchy endosperm of various cereals can digest itself even when the embryo is removed. This was demonstrated by Hansteen (15) and by Puriewitsch (30), who mounted isolated endosperms on plaster-of-Paris blocks immersed in water and found that the products of digestion were thereby drawn away and the tissue depleted. Linz (22) showed also that an increase of diastase occurred in isolated endosperms under similar conditions.

There is, however, considerable divergence of opinion as to whether these results definitely indicate that the cells of the endosperm are alive, as Hansteen and Puriewitsch claim. It was believed originally by Brown and Morris (3) that the endosperm of barley, apart from the aleurone layer, is non-living, despite the presence of diastase in its cells; and Haberlandt (14), Grüss (13) and others have attributed the diastase to a secretion from the aleurone layer. Bruschi (4) has more recently come to the conclusion that " the self-emptying can go on in the absence of any vitality in the amyliferous cells, because the starch hydrolysis is accelerated by a strong amylase which, little by little, arises from a pro-enzyme which exists in the endosperm of the resting seed, and becomes active even though every trace of vitality has been removed from the entire endosperm or seed by mechanical means." Bruschi, however, adds that the vitality characteristic of the aleurone layer is also retained in one or several of the sub-aleuronic layers.

In the light of these conclusions it was considered a matter of interest to investigate the behaviour of mitochondria in isolated maize endosperms treated as in the experiments of Hansteen and Puriewitsch. The endosperms treated in this manner have been referred to already under the previous heading.

Examination of sections stained *intra vitam* on the sixteenth day after the commencement of the experiment showed the number of mitochondria to be distinctly greater than in the resting state. They now occurred in clumps scattered through the cells in the interstices of the still intact starch grains (fig. 8), very much as in that portion of the endosperm of the normally germinating grain which has not yet begun to empty (fig. 4). Not only was this so, but in many cells also mitochondria were observed in elongated form and in process of binary fission (fig. 7). The latter observation was considered to be of great importance, since it definitely establishes the ability of mitochondria to increase in the endosperm apart from secretion from the scutellum or the aleurone cells.

At this time no corrosion of starch grains was evident. Examination on the twenty-sixth day, however, showed corrosion to be taking place slowly in the cells near the plaster-of-Paris block. Numbers of the starch grains were in process of solution in these cells, and many also had small numbers of mitochondria adhering to their surface (Plate 12, fig. 10).

It thus seems that mitochondria increase in number in the isolated endosperm and become associated with starch hydrolysis in the same manner as was observed with those secreted from the scutellum. It must be added, however, that this hydrolysing process is on a much reduced scale compared with that occurring during normal germination, both the numbers of mitochondria and more especially the rate of starch solution being decidedly less. That it does occur is nevertheless suggestive of vitality being possessed by all the cells of the endosperm, since it does not seem likely that mitochondria could divide in non-living cells.

Secretion of Mitochondria from the Scutellum in Germinating Wheat and Barley.

In order to discover whether this behaviour of the mitochondria in germinating maize was not a specific peculiarity, examination was made of germinating wheat and barley. In these cereals the metabolism is fundamentally similar to that of maize, except that there is produced, apparently from the scutellum, a cyto-hydrolytic enzyme which dissolves the walls of the endosperm cells prior to starch hydrolysis. While the observations made were not so extensive as in the case of maize, it was found that during the germination of both wheat and barley a similar abundant secretion of mitochondria into the endosperm takes place from the epithelial cells of the scutellum (fig. 10), and that these mitochondria aggregate round the starch grains prior to their solution in the manner already described.

Concluding Remarks.

It may be concluded from the observations recorded in these pages that, wherever there is indication of enzymatic activity in the solution of starch in germinating cereals, the enzyme appears to be localised within mitochondria : when the enzyme is secreted it is translocated likewise in the mitochondria and is liberated therefrom only when it has reached its substrate. It is significant also that the numbers of mitochondria produced are to some degree comparable with the extent of enzyme action taking place : in the rapid depletion of normal germination they are secreted into the starch-containing cells in considerable profusion ; in the slower auto-digestion of the isolated endosperm they multiply intracellularly to a less extent. The rate of depletion thus tends to be proportional to the surface area available from which the enzyme may be liberated ; and, while it is possible that mitochondria of endospermic origin may function to some extent in starch hydrolysis during normal germination, it is to be concluded that an important part in this process is effected by those secreted from the embryo.

In view of the continuous output of mitochondria from the epithelium of the scutellum, one must assume that there is a source of raw material for their rapid production ; and since mitochondria are largely lipoidal, one wonders whether it is the store of lipoid in the scutellum which render this production possible, and whether it is the absence of lipoids from the endosperm which prevents the same profuse propagation of mitochondria in this tissue when it is separated from the embryo. Mitochondria are believed also to contain a certain amount of protein (5); and in this connection it is curious to recall the suggestion made by Brown and Morris (3) and by Haberlandt (14) that the production of enzymes in part depends upon the absorption of proteins from the endosperm.

That mitochondria should be able to migrate through plasma-membrane and cell-wall in the manner described is not to be considered in any way unfeasible. It is generally concluded (Cowdry, 5) that mitochondria consist for the most part of phosphatides with a small amount of protein in addition; and there is sufficient evidence for believing that phosphatides can migrate through tissue in this manner. For example, it is known that fatty substances of various kinds pass from the apical meristems where they are synthesised out into the external differentiating tissues (Priestley, 29), in which translocation they pass through numerous cell-walls; more specific evidence, however, lies in the experiments of Hansteen Cranner (8) and Grafe and Magistris (11), who demonstrated that phosphatides can diffuse out from discs of plant tissue when these are allowed to stand in water. In view of this knowledge the translocation of such minute droplets of phosphatide material as mitochondria should not be difficult of conception.

It is premature as yet to speculate upon the mechanism of the relationship of mitochondria to the manifold metabolic processes in which they seem to be concerned: Robertson has, nevertheless, shown how the physical properties of their lipoid components may be of fundamental significance; and, if indeed they consist mainly of phosphatides, then in chemical reactions of various kinds taking place at their surface, and perhaps in the holding and release of enzymes, much may depend on the ethylene linkages of their unsaturated fatty-acid radicles (Mayer, Rathery and Schaeffer, 23) and their amphoteric properties recently demonstrated by Levene (21).

Summary.

In the resting stage of the maize grain mitochondria occur in moderate numbers in the scutellum and occasionally in the endosperm. During germination, however, they become more numerous in the scutellum and are secreted in large numbers from the epithelial cells into the adjacent starch-containing cells of the endosperm. These secreted mitochondria then aggregate round the starch grains prior to their corrosion; then as corrosion commences the associated mitochondria seem to disappear. Throughout the whole period

of endosperm depletion, mitochondria are secreted in this manner and migrate through the emptied cells to the zone of active hydrolysis, where they become associated with the starch grains in this manner.

This behaviour of the mitochondria appears to lend considerable support to the enzymatic conception of mitochondrial activity, it being supposed that the starch-splitting enzyme is located within them or at their surface, and is liberated therefrom when these bodies have reached the surface of the starch grains.

During germination the mitochondria originally present in the endosperm are able to increase by fission, although the same profuse numbers are not found as are secreted from the scutellum. In isolated endosperms, however, these mitochondria of intracellular origin are able to effect starch hydrolysis and depletion, although at a much lower rate corresponding to their lesser numbers.

There is no evidence for a secretion of mitochondria from the aleurone layer or of depletion being in any way affected by secretion of enzyme therefrom.

A similar secretion of mitochondria from the epithelial cells of the scutellum takes place during the germination of both wheat and barley.

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DESCRIPTION OF PLATES.

PLATE 7.

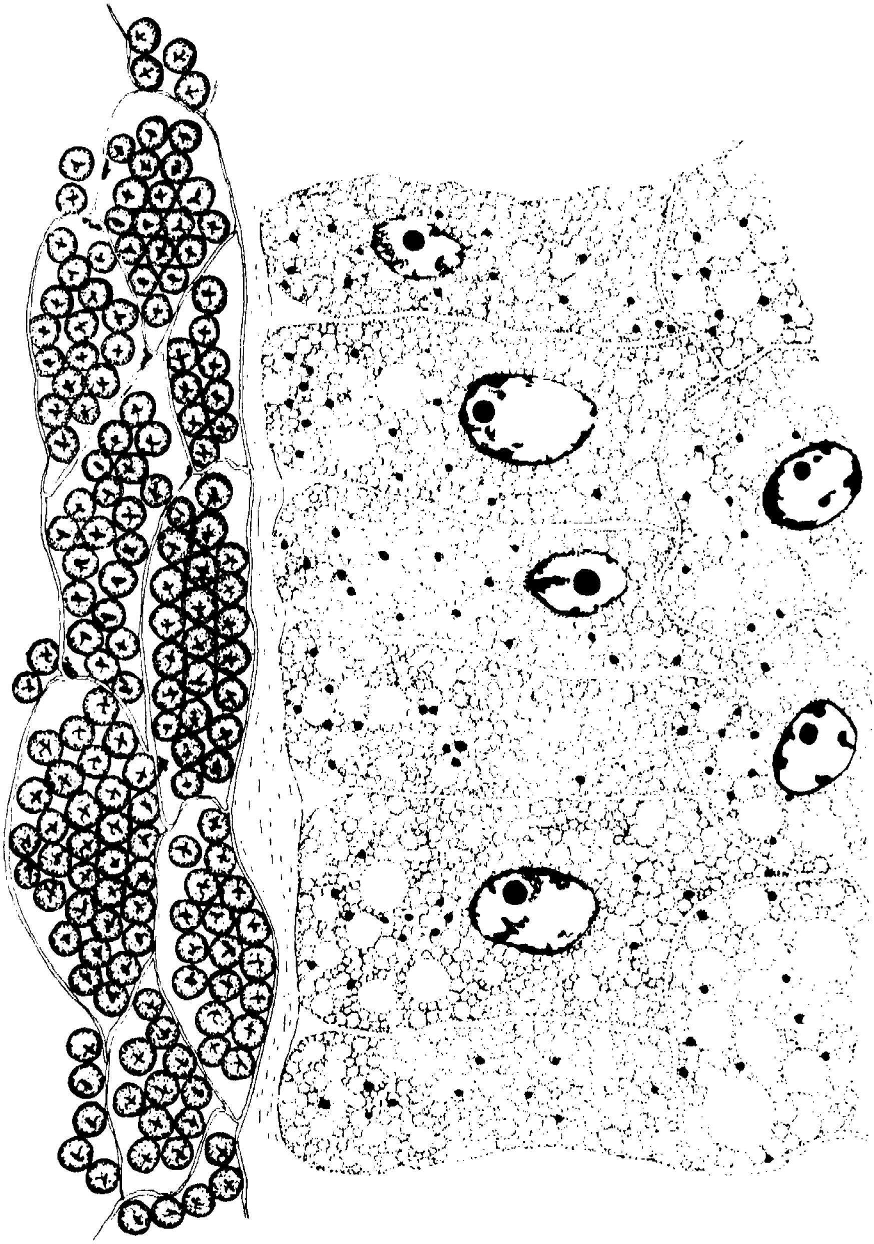
FIG. 1.—Portion of a transverse section of a resting grain of maize, fixed in osmo-chromic solution and stained with iron hæmatoxylin, showing scutellar epithelial cells and adjacent endosperm cells. In the epithelial cells and the portion of the tissue of the scutellum showing above them, are seen the large nuclei with characteristic peripheral chromatin described by Reed (31), and also numerous vacuoles. Below the epithelium is the layer of crushed cell-walls characteristic of the edge of the endosperm, followed by the cells packed with starch grains. Mitochondria are in comparative abundance in the scutellum, but only a few can be observed in the endosperm cells. Numerous brown-stained lipoidal droplets were seen in the protoplasm, but these have been omitted from the drawing to prevent confusion with the mitochondria. (× 1200.)

PLATE 8.

FIG. 2.—Portion of a transverse section of a grain of maize, fixed on the fifth day of germination in Cox's solution, showing a portion of the scutellar epithelium. In both the epithelial cells and the inner tissue of the scutellum partly seen above them, the mitochondria are now much more numerous than in the resting stage depicted in fig. 1. They are also clustering on the exterior of the epithelial cells and partly embedded in their walls, thus appearing as if they had just been secreted. Mitochondria are seen also in the intervening space between the scutellum and the endosperm and clustering among the crushed cell-walls bounding the endosperm itself. (× 1200.)

PLATE 9.

FIG. 3.—Portion of a transverse section of a maize grain fixed in Champy-Kull solution on the sixth day of germination. Mitochondria are seen accumulated at the peripheral ends of the epithelial cells and in process of passage into the adjacent endosperm. Some are embedded in the mass of crushed cell-walls bounding the endosperm, and in the cells immediately within they occur in profusion. The more peripheral



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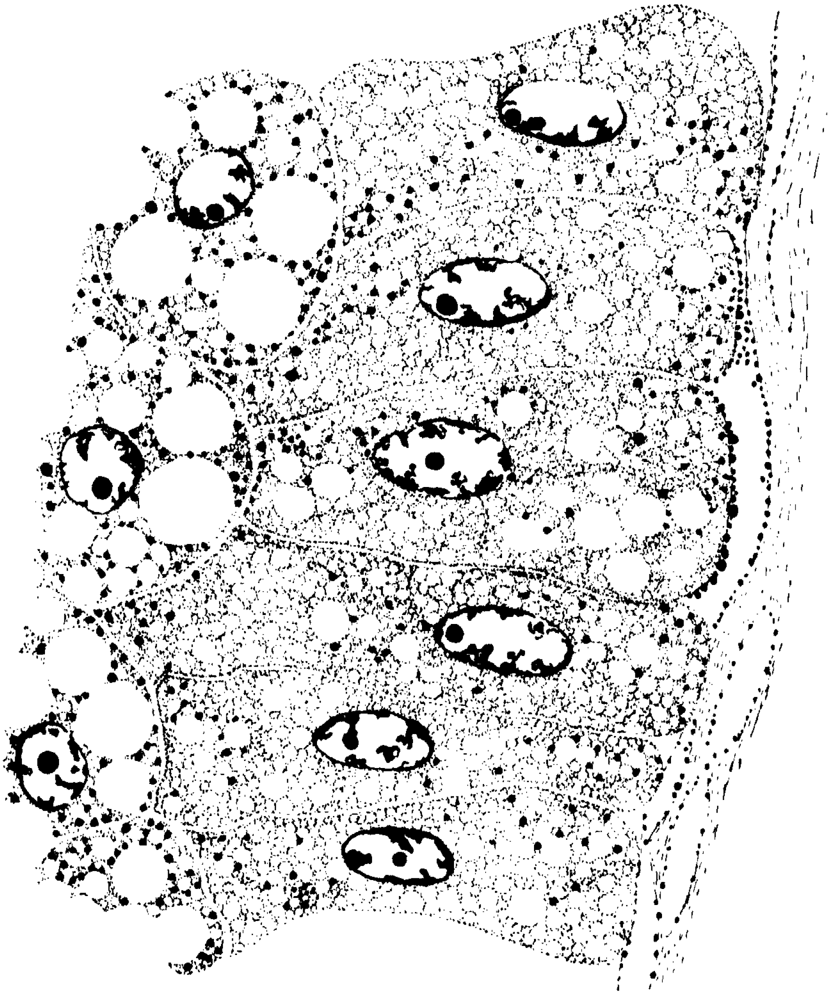


Fig. 2.

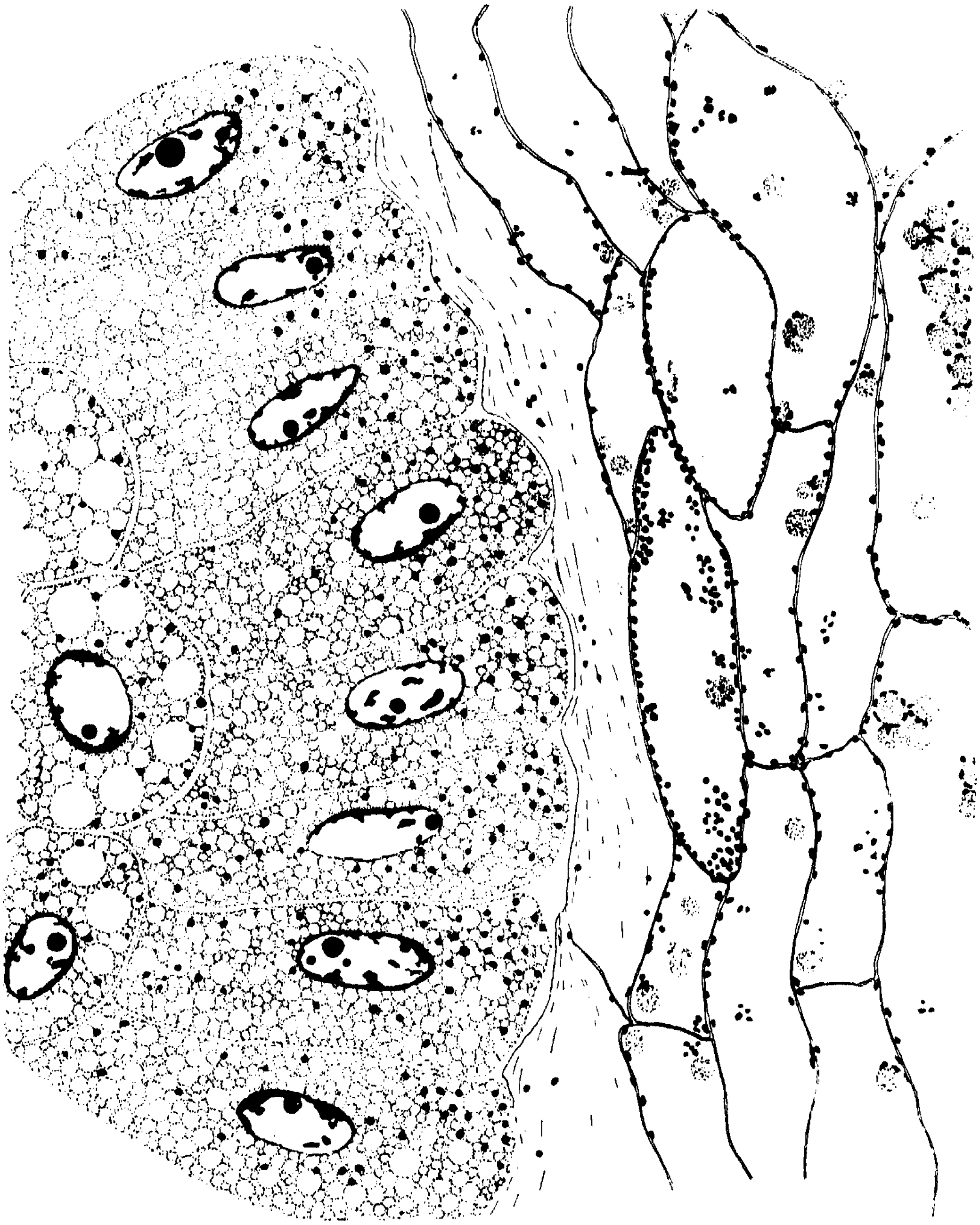


Fig. 3.

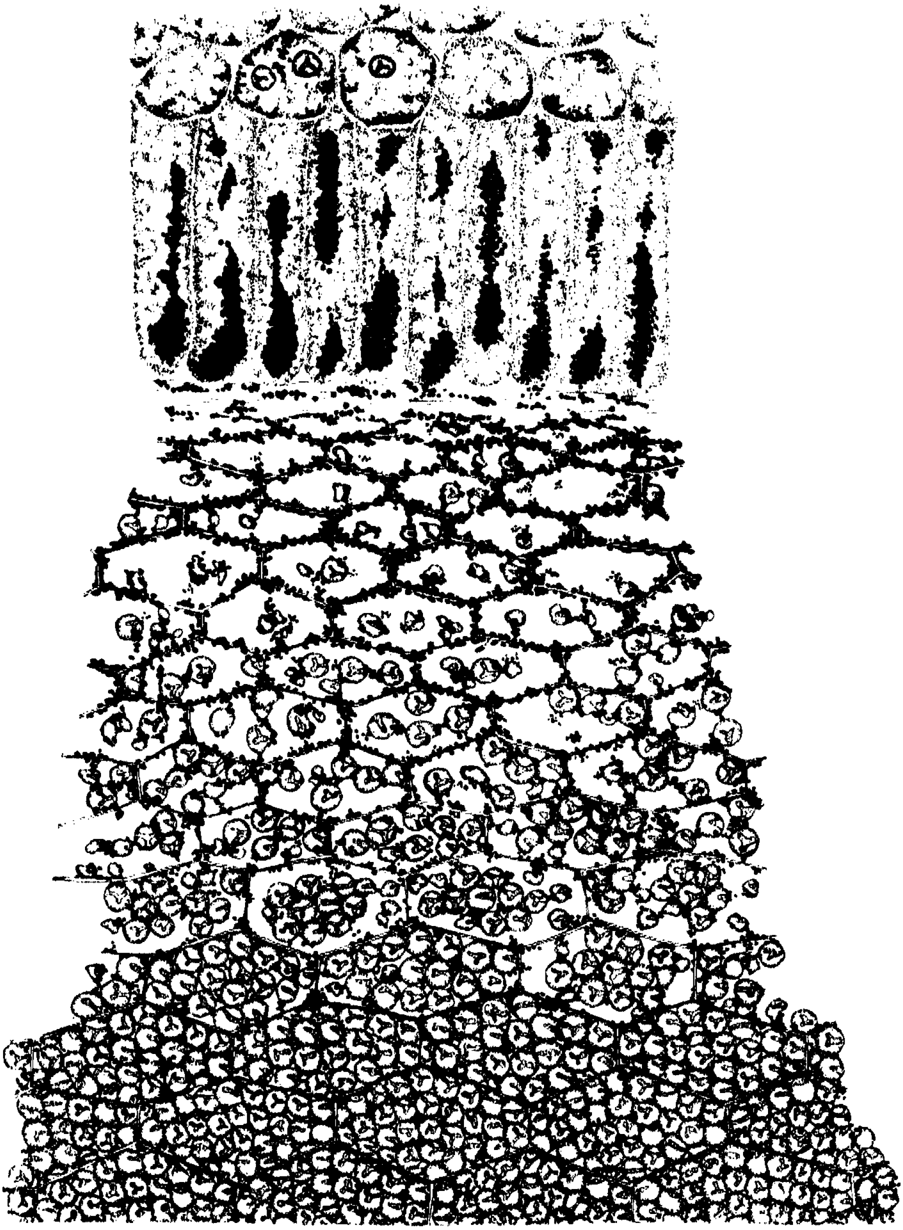


Fig. 4.

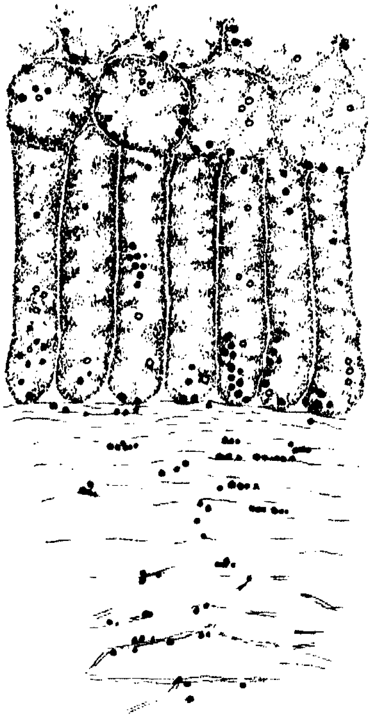


Fig. 5.

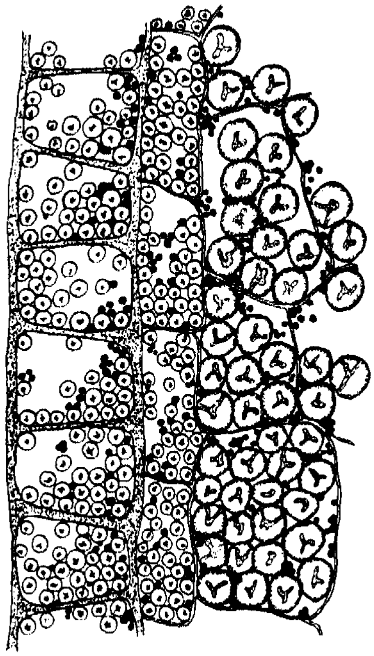
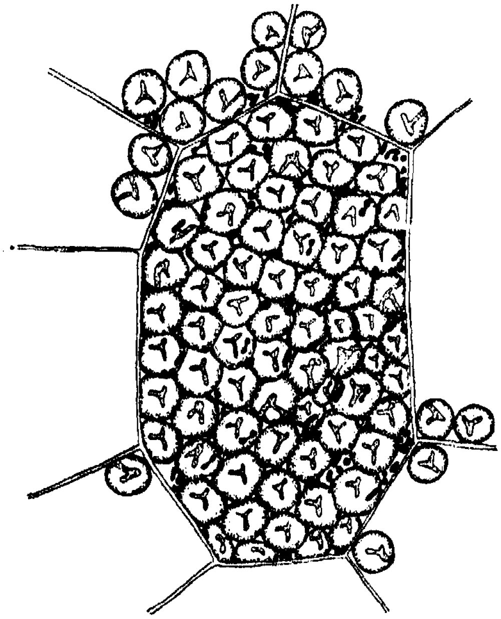


Fig. 6.



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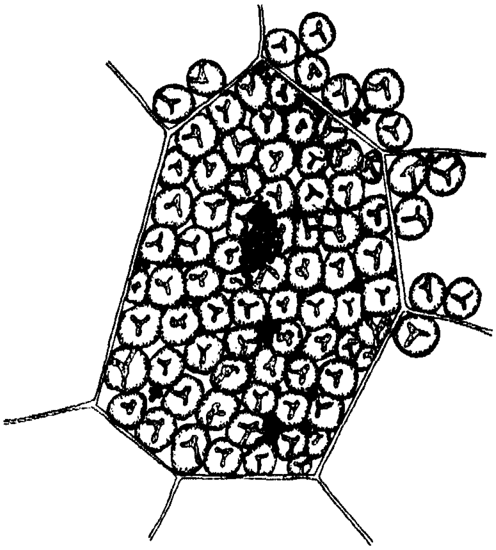

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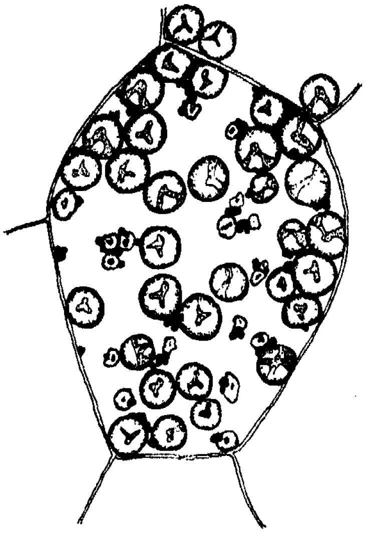

FIG. 9.

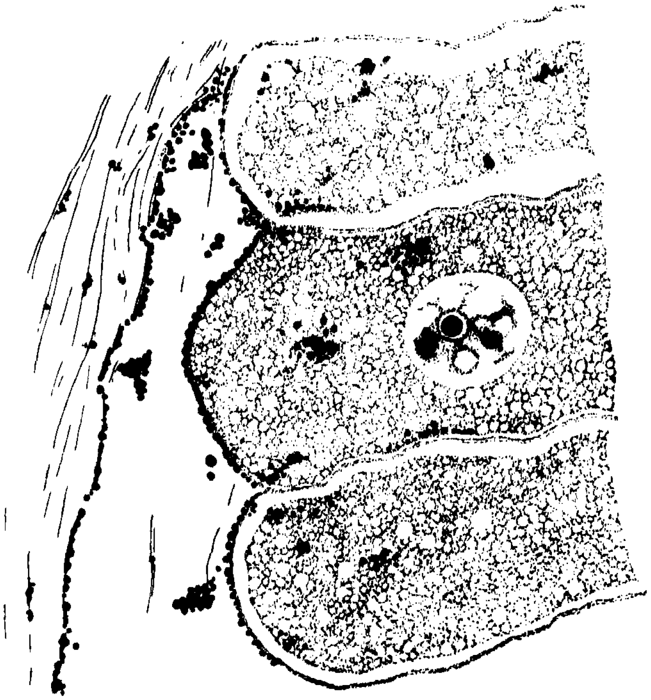

FIG. 10.

endosperm cells are practically depleted except for a few starch grains in process of solution and show numerous mitochondria accumulated for the most part round their walls. The cells farther in contain more starch grains which have not yet commenced to corrode, and round these mitochondria are clustered in numbers; starch grains which are in process of corrosion are, on the contrary, generally free from mitochondria. (× 1200.)

PLATE 10.

FIG. 4.—Section of maize grain, eight days after the commencement of germination, stained *intra vitam* with Janus green B, showing secretion of mitochondria from the scutellum into the endosperm. Mitochondria are seen in dense profusion both in the epithelial cells and in the adjacent crushed mass of endosperm walls; they are seen also in numbers in the cells undergoing depletion, as in fig. 3, some being aggregated round the starch grains and others being clustered along the cell walls. It will be observed how the number of mitochondria decreases on passing towards the undepleted portion of the endosperm. Occasional starch grains are seen in the tissue of the scutellum. (× 600.)

PLATE 11.

FIG. 5.—Portion of section of maize stained *intra vitam* on the fourteenth day of germination, at which stage endosperm depletion is practically completed. The now considerably elongated epithelial cells are beginning to clear of mitochondria with the approaching cessation of secretory activity; and the layer of crushed endosperm cell-walls has become very broad with the continued depletion and concomitant expansion of the scutellum. Mitochondria, however, are still in progress of passage through this zone to the not yet depleted cells beneath. (× 640.)

FIG. 6.—Drawing of a portion of the aleurone and sub-aleuronic layers in maize grain on the tenth day of germination. The preparation was stained *intra vitam* with Janus green B. Protein granules are seen in the aleurone cells and in the adjacent layer, while starch grains occupy the cells farther in. Mitochondria are present in the aleurone cells, and in the adjacent endosperm cells they occur in numbers characteristic of the whole of this tissue not undergoing depletion at this stage. There is no suggestion of secretion from the aleurone cells or of the profusion of mitochondria found to accompany secretion from the epithelial layer of the scutellum. (× 1300.)

FIG. 7.—Portion of a section of an experimentally treated isolated maize endosperm, sixteen days after commencement of the experiment, and stained *intra vitam* with Janus green B. Mitochondria are seen in the elongated state and undergoing fission among the densely packed starch grains. (× 1300.)

PLATE 12.

FIG. 8.—Another cell in the same section as illustrated in fig. 7, showing a cell where the mitochondria are occurring in clumps. It is considered probable that these clumps have arisen from the small number of mitochondria present in the resting stage by division such as is illustrated in fig. 7. (× 1300.)

FIG. 9.—Portion of a transverse section of another isolated maize endosperm, twenty-six days after the commencement of the experiment. The cell depicted was quite near the plaster-of-Paris pillar, and is undergoing depletion. Some of the starch grains are seen to be corroding, and some have small numbers of mitochondria aggregated

round them, showing that the mitochondria of endospermic origin, like those secreted from the scutellum, only in a smaller way, are associated with starch hydrolysis. (× 1300.)

Fig. 10.—Portion of a transverse section through a germinating wheat grain five days old. The material was fixed in osmo-chromic solution. The apical portion of the epithelial cells of the scutellum are seen and also the adjacent crushed layer of endosperm cell-walls. Mitochondria are seen to be in process of secretion from the epithelium precisely as in the case of maize. (× 1300.)

The Development and Morphology of the Gonads of the Mouse. Part II.—The Development of the Wolffian Body and Ducts.

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(Communicated by Prof. J. P. Hill, F.R.S.—Received August 4, 1927.)

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[Plates 13–16.]

CONTENTS.

Page
1. Introduction 206
2. Material 207
3. Description 208
4. Discussion 217
5. Summary, Bibliography, etc. 219

1. *Introduction.*

This paper is concerned with the embryonic development of the Wolffian body and duct, the Müllerian duct, and the urogenital connection in the mouse.

Part I dealt with the development of the indifferent gonad and of the ovary after differentiation. The germinal ridge is first established in the embryo 9 days *p.c.*, and the gonads are definitely differentiated into ovaries or testes 12 days *p.c.* Parturition takes place 19 days *p.c.*, and sexual maturity is attained at about 8 weeks *p.p.*

In the mouse the mesonephros is vestigial and non-functional, a point that must be constantly borne in mind in comparing it with, for instance, such forms as the rabbit, in which the mesonephros is large, well developed and functional.

The epithelial proliferation beginning in the forming germinal ridge and continuing, in the female, until after birth is not in the form of cords, but is continuous over the whole surface of the gonad, and gives rise to the solid epithelial nucleus. Furthermore, it is not divided into a medullary and a cortical proliferation, but is one single process. After the ovary is differentiated the epithelial nucleus is broken up, to a certain extent, into finger-shaped lobes or cords by the down-growth of mesenchyme from the hilum, but this process is entirely secondary.

In the male the epithelial proliferation and the solid epithelial nucleus continue until the tunica albuginea is formed at the time of sexual differentiation. Mesenchyme growing down from the hilum forms the tunica albuginea, and breaks up the solid epithelial nucleus into spermatic cords. The formation of the spermatic cords is, therefore, also secondary. These peculiarities of the mouse are probably associated with the small size and rapid development of the early gonad rudiments. They are of the utmost importance in connection with the relations of the urogenital connections and ducts to the spermatic tubules in the testis and the homologous structures in the ovary.

The author would like to take this opportunity of expressing his thanks to Prof. J. P. Hill, F.R.S., for his advice and criticism in connection with this work, and to Dr. A. S. Parkes for much helpful criticism. The material for this research was purchased with the help of a grant from the International Education Board. The histological and photomicrographic expenses were defrayed by a grant from the Government Grants Committee of the Royal Society. I wish to express my thanks for both grants.

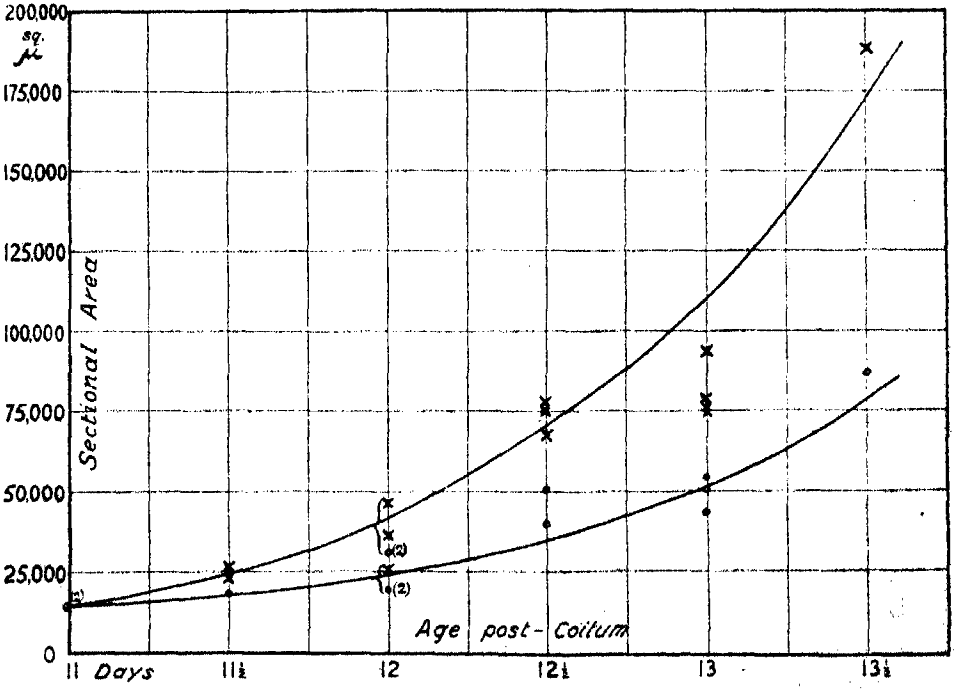
2. *Material.*

The material available consisted of 19 embryos from 9 to 12 days *p.c.*, arranged in 9 stages, as the variation in the state of development necessitated a seriation not strictly in accordance with their ages. The material after 12 days *p.c.* was taken at daily or half-daily intervals until birth. It consisted of 18 female embryos arranged in 8 stages and of 21 male embryos in corresponding stages. The series of material from birth to maturity consisted of 11 stages of each sex, covering each of the eight weeks.

The material was mostly fixed in Bouin's fluid and stained with Ehrlich's hæmatoxylin and eosin, or by Pasini's method. Complete serial sections were made of the gonads or gonad regions in each case, excepting the larger *p.p.* testes. In addition, embryos at 12, 12¼, 12½, 15 and 18 days *p.c.* were dissected under a dissecting microscope.

3. *Description.*

The Differentiation of Sex.—Sex can first be distinguished histologically in the mouse at 11½ or 12 days *p.c.* (5). It can be determined on micro-dissection easily at 13 days *p.c.* by the greater thickness of the testes as compared to the ovaries. By comparing micro-dissections of embryos from the same uteri, it was possible to distinguish the testes from the ovaries at 12½ and 12¼ days *p.c.* by the same method. At 12 days this distinction was not apparent. Measurements were made from the slides of the lengths of all the gonads of the embryos in the series between 11 and 13½ days, inclusive. At the same time an outline drawing of the greatest cross-section of each was made with the aid of a camera lucida. The lengths of the gonads varied greatly, and were not correlated with sex. The area of the cross-sections was calculated with the aid of a planimeter, and the average of the two gonads of each embryo was plotted on the accompanying graph (text-fig. 1). It can be seen from this that the



TEXT-FIG. 1.

testes are perceptibly thicker at 11½ days, and are increasingly so at all subsequent stages. In no case after sex can be distinguished histologically do the cross-sectional areas of the ovaries of any of the females overlap those of the testes of the males from the same uterus. The apparent overlap at 12 days is not real, as the embryos from two uteri are plotted. Those from one uterus (Stage 7) are less developed histologically, and are all smaller than any of those from the second uterus (Stage 9). Drawings of micro-dissections of male and female 12½ day *p.c.* embryos are shown in Plate 13, figs. 1 and 2. Similar dissections at 15 days *p.c.* are shown in Plate 14, figs. 1 and 2. In the testes the loops of the spermatic cords, attached to the mesotestis, can be seen as transverse ridges through the tunica albuginea. It is remarkable that the testes can be distinguished by measurement at the earliest stage at which they can be identified histologically, and that they can be told 24 hours later under a dissecting microscope or even with the naked eye.

The Wolffian Body and Duct at the Time of the Formation of the Germinal Ridge.—This description begins with embryos of 9 days *p.c.* at the stage when the first traces of the germinal ridge are forming. The development (and degeneration) of the Wolffian body, the Wolffian duct and the Müllerian duct always proceeds strictly in an antero-posterior direction. In consequence, the stage of development of any one of these organs is usually noticeably more advanced at the anterior end than at the posterior end. In embryos 9 days *p.c.* (Stage 1 (5)) the Wolffian body is well established, and the tubules are formed anteriorly. The most posterior tubules are, however, not quite completely organised. The Wolffian duct is luminate throughout its length.

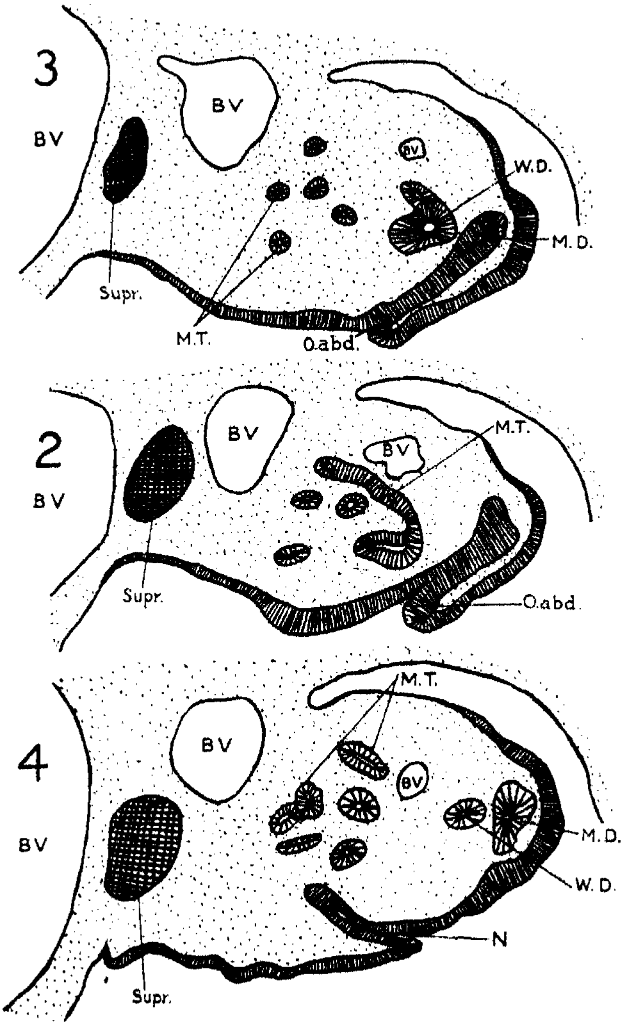
Anteriorly it ends blindly just in front of the opening into it of the first mesonephric tubule. Posteriorly it opens into the urogenital sinus. It measures approximately 35 to 40 μ in diameter. It may therefore be said that the Wolffian bodies and ducts are completely established at the time when the germinal ridges begin to form. Growth, however, continues in both for some time afterwards. At the next stage (11 days *p.c.*, Stage 3) 8 to 10 mesonephric tubules are found on each side. They are simple, slightly coiled tubules about 55 to 90 μ in diameter, with a small but definite lumen. Their wall is formed of a single layer of columnar epithelial cells with the nuclei away from the lumen. The tubules are without definite glomeruli, which are never developed, but rudimentary capsules in the form of slight swellings of the tubules are found in 11-day *p.c.* embryos.

All the anterior tubules open into the Wolffian duct at regular intervals, but the posterior two or three do not, and are blind at both ends. There is no separation of the anterior and posterior tubules into groups, but it is possible that the anterior ones, opening into the Wolffian duct, represent the epigonal and the blind tubules the paragonal regions. The first tubules are slightly anterior to the epithelial nucleus of the gonad, and there are no tubules posterior to the anterior two-thirds of the latter. Each tubule ends close to the peritoneal epithelium on the ventro-lateral aspect of the germinal ridge, the ends of those in the region of the gonad being only separated from the dorso-lateral border of the epithelial nucleus by a thin layer of mesenchyme. The extremities of the anterior tubules appear to be attached to the cœlomic epithelium as nephrostomes, but it is impossible to be sure, in the single specimen of this stage, on account of the plane of sectioning being longitudinal, not tangential. Nephrostomial connections between the anterior tubules and the cœlomic epithelium are present in the embryo 10 days *p.c.* (Stage 5 (5)).

Small funnel-shaped invaginations of the cœlom are present in the most anterior nephrostomes, which are best developed. Three or four nephrostomes are anterior to the gonad, and a few, more difficult to distinguish, are present at the dorso-lateral margin of the anterior portion of the gonad. At 11 days *p.c.* (Stage 6 (5)) better developed nephrostomes are present. About 3 occur anterior (Plate 15, fig. 1) to the gonad and 3 at least in the gonad region (Plate 15, fig. 2). In one embryo, a typical example, the ostium abdominale tubæ (text-figs. 2 and 3) occurred 182 μ in front of the gonad. The first nephrostome (text-fig. 4) was 84 μ posterior to the ostium, the second 102 μ, and the third 154 μ, or 28 μ anterior to the gonad. The fourth was 21 μ posterior to the anterior pole of the gonad, the fifth 63 μ, and the sixth 147 μ. Traces of two more nephrostomes occurred behind the sixth. The two or three posterior tubules, which do not open into the Wolffian duct, have no nephrostomial connection with the cœlomic epithelium.

In the clearest cases the nephrostomes are open and appear continuous with the lumina of the tubules; in others they consist merely of solid connections between the peritoneal epithelium and the tubules. The ventral borders of the nephrostomes in the gonad region, immediately beneath the cœlomic epithelium, are either in contact with the dorsal margin of the epithelial nucleus of the gonad or are only separated by a very thin layer of mesenchymal tissue. These nephrostomes are destined to participate in forming the rete testis or the rete ovarii, as the case may be. They remain unchanged in both sexes until 12 days *p.c.*

It has already been stated that glomeruli as such are absent in the mesonephros of the mouse, but rudimentary capsules of Bowman are represented by a slight swelling of the distal end of each tubule just before it joins the nephrostome. This swelling is slight but distinct, and its lumen is a little larger than that of the tubule just above it. Its wall is similar to the rest of the tubule.



TEXT-FIGS. 2-4.

(× 135. See description of Plates for key to reference letters.)

The cephalic portion of the Wolffian body anterior to the gonad becomes much reduced by the 12th day *p.c.*, and hardly projects at all in front of it. This reduction is partly apparent, owing to the unequal and relatively enormous growth of the gonal region, and partly real, owing to atrophy of the pregonal region. In consequence, at the time when the urogenital union is effected, all the nephrostomial rudiments are grouped about the anterior end of the gonad, and the ostium abdominale tubæ is just in front of the anterior pole of the gonad.

The Urogenital Union of the Male.—In the male the urogenital connection is effected slightly earlier than in the female. In the male embryo 12 days *p.c.* (Stage 9 (5)) the nephrostomes have ceased to communicate with the cœlom, and the nephrostomial canals are becoming separated by mesenchyme from the cœlomic epithelium. At the same time the epithelial nucleus is becoming differentiated into definite spermatic cords by the down-growth of mesenchymal tissue from the primitive hilum. These spermatic cords are attached along the line of the hilum. The nephrostomial canals lie in close apposition to the hilar insertions of the forming spermatic cords, and are only separated from them by a thin layer of mesenchyme. As the nephrostomial canals become separated from the cœlomic epithelium they unite with the spermatic cords and constitute the urogenital connection.

The actual union seems to be effected before the nephrostome is completely separated from the cœlomic epithelium by a small down-growth from the ventral surface of the nephrostomial canal, near its junction with the epithelium (Plate 15, fig. 3). This down-growth breaks through the thin layer of mesenchyme, separating it, and fuses with the spermatic cords. This connecting strand is small, and consists of only a few cells, as can be seen from the figure. It appears to be formed by a definite down-growth from the nephrostomial canal just distal to its cœlomic opening. At this stage the rudimentary capsules of Bowman are so degenerate as to be indistinguishable from the nephrostomial canals on the one side and the mesonephric tubules on the other. It is thus impossible to define accurately the extent of the remains of the capsules. In consequence, it is possible that cells which previously formed part of a Bowman's capsule may enter into the formation of the strand of cells which grows down from the nephrostomial canal and unites with the spermatic tubules. It would appear more probable, however, that the strand is formed as described from the elements of the nephrostomial canal alone.

The union is assisted by the constriction of the primitive hilum to form the mesorchium. This is effected by the formation of a lateral groove between the Wolffian body and the dorsal margin of the testis, which deepens and displaces the nephrostomial canals from the lateral margin towards the mid-line of the testis, and brings them into closer contact with the dorsal extremities of the spermatic cords.

The primitive nephrostomial canals, together with small down-growths from them, therefore appear to give origin to the definitive rete testis and, by acquiring an early, but secondary, connection with the spermatic cords, unite them to the anterior mesonephric tubules which open into the Wolffian duct. The posterior tubules, which never developed nephrostomial rudiments, do not contribute to the urogenital connection. The rete testis consists of a bundle of about seven fine tubules running from the testis through the anterior portion of the mesorchium to the anterior end of the Wolffian body. The lumina of the rete tubules remain incipient for some time, but become more marked by 14 days *p.c.* and are open at birth.

It appears that all the six to eight nephrostomial canals that are formed take part in the urogenital union. They are at first separate and distinct. Soon the constriction of the mesorchium presses them closer together and they then anastomose, forming a closely interwoven cord extending forward from the testis through the anterior margin of the mesorchium to the cephalic or epididymal portion of the Wolffian body. The increase in the number of spermatic cords results in the ends of the rete becoming branched to unite with them.

These branches constitute the straight tubules of the testis, the tubuli recti. They are formed in part from the rete, but may be contributed to also by the hilar ends of the spermatic tubules. They do not exist as such at the time when the urogenital union is effected, but are developed before birth to deal with the increased number of spermatic tubules. They arise thus as a secondary differentiation of the region of junction between the spermatic tubules and the rete. At 18 days *p.c.* their lumina are well formed and much larger and more distinct than those of the rete. At this time the spermatic tubules are not yet luminate.

The connective-tissue sheaths of the spermatic tubules, which were derived from the down-growth of mesenchyme from the hilum, unite to form a sheath around the tubuli recti and rete. This sheath is augmented by the continued down-growth of connective tissue from the hilum and forms the mediastinum testis.

The vessels of the testis pass through the mesorchium immediately behind the rete cords (Plate 16, fig. 1).

The Urogenital Union in the Female.—The urogenital connection in the female is established slightly later than in the male. It is effected in an exactly similar manner. The female embryo at 12½ days *p.c.* is at the same stage in this respect as the male embryo at 12 days *p.c.* At this stage the small ventral down-growths from the nephrostomial canals are passing into continuity with the epithelial nucleus of the gonad, and the nephrostomes are looing their connection with the cœlomic epithelium. This process, as in the male, is slightly more advanced in the anterior nephrostomial canals than in the more posterior ones. At this time the mesenchyme of the hilum is beginning to grow down into the epithelial nucleus and to split it up into epithelial cords. These down-growths constitute the primordia of the connective tissue of the septa ovarii. At 13½ days *p.c.* the septa have extended from the hilum as far as the periphery of the ovary. The division of the ovarian epithelial nucleus into epithelial cords is, therefore, like the formation of the spermatic cords in the male, a secondary process. The retia primordia effect their union with these epithelial cords at the time when they are just forming. The urogenital union in the female mouse is thus effected in a manner strictly homologous to that in the male. The constriction of the mesovarium, by the formation and deepening of a lateral groove along the line of junction of he ovary with the Wolffian body, assists in bringing about the urogenital union, in the same manner as the constriction of the mesorchium in the male.

The primitive nephrostomial canals and the down-growths from them therefore give origin to the definitive rete ovarii and unite the epithelial cords to the anterior mesonephric tubules. At the time when the union is effected the anterior mesonephric tubules open into the Wolffian duct. The posterior tubules, which, as in the male, never develop nephrostomes, do not contribute to the urogenital connection. The rete ovarii thus consists primarily of a bundle of about eight fine tubules running from the ovary through the anterior portion of the mesovarium to the anterior end of the Wolffian body. The lumina remain incipient until about the 18th day *p.c.*, when they become open.

It appears that, as in the male, all the nephrostomial canals take part in the formation of the rete. They are at first separate and distinct, but soon anastomose with each other.

The hilar portions of the septa ovarii, augmented by a further down-growth of mesenchymatous connective tissue from the hilum, ensheath the ovarian portion of the rete and their junctions with the epithelial cords. This forms the mediastinum ovarii, the homologue of the mediastinum testis.

The vessels of the ovary pass through the mesovarium immediately behind the rete cord (Plate 16, fig. 2).

The Later Development of the Wolffian Body and Duct in the Female.—The down-growth of connective tissue from the hilum which contributes to the formation of the mediastinum ovarii continues, and forms a central knob of tissue in the ovary by the end of gestation. This knob of tissue constitutes the primitive medulla. It includes the ovarian portion of the rete. As development proceeds the medulla is contributed to by the inner ends of the epithelial cords, in which the germ-cells have degenerated. The rete tubules loose their definite connections with the epithelial cords of the definitive cortex during this process of medulla formation. They persist, however, during adult life as a number of isolated cords in the medulla.

In the female the Wolffian duct degenerates early. At 14 days *p.c.* the anterior end shows signs of degeneration, at 15 days *p.c.* it has degenerated throughout the ovarian region. The degeneration proceeds from before backwards.

The anterior mesonephric tubules, about 8 in number, which at first unite the rete with the Wolffian duct, constitute in the female the epoophoron (Plate 16, fig. 2). They loose their connection with the Wolffian duct as soon as it begins to degenerate. Subsequently they persist, without developing further, as a few blind tubules to which the rete tubules are attached, and which can be detected in the mesovarium of the adult female.

The blind posterior tubules, one or two in number, which do not take part in the urogenital connection, constitute the paroophoron. Like the paradidymis in the male, it degenerates early and cannot be detected at birth.

The Early Development of the Müllerian Duct.—There are no traces of the ostium abdominale tubæ to be found in the embryos earlier than 10 days *p.c.* (Stage 5 (5)). At 10 days *p.c.* a varying number of rudimentary nephrostomes in connection with the nephric tubules are to be found in the region where the ostium will be developed. It is impossible to distinguish the primordium of the ostium from the other nephrostomes. At the next stage (Stage 6 (5)), 11 days *p.c.*, the ostium can be distinguished from the nephrostomes. It consists of a groove or funnel in the ventral side of the urogenital fold, and is situated on a level with the extreme anterior end of the Wolffian duct. Except for its somewhat larger size and the absence of any connection with the mesonephric tubules, it closely resembles in structure and position the true nephrostomes. It is 84 μ anterior to the first nephrostome and 182 μ anterior to the gonad. At 12 days *p.c.* (Stage 9 (5)) the ostium abdominale tubæ has deepened. Its free extremity extends up beneath the lateral epithelium of the urogenital fold almost to the level of the Wolffian duct, and proceeds to grow backwards beneath the peritoneal epithelium immediately superficial to the Wolffian duct.

The rapidly growing tip exhibits many mitoses. It is closely applied to the overlying peritoneal epithelium in a manner which suggests that the latter is adding to it by proliferation. The absence of mitoses in this region of the peritoneal epithelium, however, precludes this possibility. The rapid backward growth of the tip of the ostium abdominale tubæ to form the Müllerian duct continues until it extends into the genital cord, which has been formed already by the union of the two urogenital folds. This origin is exactly similar to that described in the human embryo by Felix (7). He says: "Its growth results entirely from its own forces, the outgrowing tip being always free, and connected neither with the cœlomic epithelium nor the primary excretory duct; only poorly preserved or poorly fixed preparations could deceive one on this point. The growth depends on the increase of cells partly along the entire extent of the duct, as is shown by the mitoses, partly at the outgrowing end, which is frequently swollen and presents all the peculiarities of a so-called cone of growth."

The development of the Fallopian tubes and the ovarian capsules in the mouse is described in detail by Agduhr (1).

The Degeneration of the Müllerian Duct in the Male.—In the male the Müllerian duct degenerates early. Degeneration begins in the anterior end and proceeds caudalwards. At 12½ days *p.c.* the development of the Müllerian duct in the male is exactly similar to that in the female, and the development of the posterior region continues in the same manner until 13½ days *p.c.* Degenerative changes are first observable in the cephalic end of the duct, opposite the anterior pole of the testis, at 13 days *p.c.* At 13½ days *p.c.* the ostium abdominale tubæ is reduced to a mere blind groove, and the anterior portion of the Müllerian duct has disappeared. Farther back, opposite the posterior half of the testis, the duct is present, but is small and shows signs of degeneration. Posterior to the testis the duct is at the same stage of development as in the 13½-day *p.c.* female embryo. The Wolffian duct is, however, larger and better developed throughout its length than the Müllerian duct.

At 15 days *p.c.* the degeneration of the Müllerian duct in the male is more advanced, and at 16 days *p.c.* it has almost disappeared in the testis region. The mesenchyme around the Müllerian duct which forms the ridge that, in the female, would develop into the muscle layers of the uterus, degenerates also. It first becomes arranged loosely around the duct and then gradually disappears. The degenerative changes set in so early in the male that the anterior end of the duct has disappeared before the posterior end has reached its maximum development.

4. *Discussion.*

Many views have been put forward regarding the origin of the rete in both ovary and testis. A large number of workers, including de Winiwarter and Sainmont in the cat (11), and de Winiwarter in the rabbit (10) and man (12), consider that outgrowths from the walls of the Malpighian corpuscles unite with the sex-cords and give origin to the rete tubules.

Other workers, including Allen (2) in the pig and rabbit, and in Chrysemys (3) trace the rete to peritoneal invaginations. Allen (3) states that the rete in Chrysemys is formed by the union of " funnel-cords " (nephrostomial canals) with evaginations from the capsules of Bowman. The funnel-cords are derived from the " peritoneal funnels " (nephrostomes) of the Malpighian corpuscles. He is inclined to consider the funnel cords as modified sex-cords. Fraser (8), in the Marsupials, describes the origin of the rete from a core of cells lying within the genital ridge anterior to the germinal rudiment. She considers that in Perameles, and probably also in Trichosurus and other marsupials, the core of cells within the genital ridge is derived from the nephrostomial canals of the mesonephros, which loose their connection with both the cœlomic epithelium and the Malpighian corpuscles, and unite into a continuous mass of cells. Reference should be made to this paper for a bibliography of the subject. Van Beek (4) also traces the rete to the pre-gonal germinal ridge.

Felix (7), and recently Wilson (9), consider that the rete in the human subject originates from the deeply lying cells of the early gonad. This rete blastema, therefore, is of cœlomic epithelial origin. The rete, on this view, is not derived from the Wolffian body, and the union with the Malpighian corpuscles, or evaginations from them, is secondary. De Burlet and de Ruiter (6) have maintained that, in the mouse, the rete is formed from the longitudinal core (*Längstamm*), to which the spermatic tubules are attached, and the epithelial ridge (*Epithelkamm*) attached to it. Agduhr (1), however, maintains that, in the mouse, the rete is formed by the down-growth of epithelial cords from the cranial end of the genital ridge.

The material described in this paper shows that a nephrostomial funnel is formed in connection with each of the mesonephric tubules, which will ultimately form part of the epididymis or epoophoron. These funnels, open or closed, are situated immediately dorso-lateral to the anterior portion of the gonad, in the stage immediately before the urogenital connection is established. The end of the mesonephric tubule with which the funnel unites is sometimes slightly swollen to form a rudimentary capsule of Bowman, but no glomerulus is formed in connection with it. At the next stage the capsules of Bowman are difficult to define, and more or less indistinguishable from the nephrostomial canal on the one side and the mesonephric tubule on the other. The nephrostomial canals are becoming detached from the peritoneal epithelium, and at the same time they are united with the epithelial nucleus of the gonad by strands of cells. These strands appear to grow down as a solid evagination from the ventral side of the nephrostomial canal at a point close to its cœlomic extremity.

If these strands of cells are formed from the elements of the degenerate capsules of Bowman, the condition in the mouse is homologous to that described in other animals by de Winiwarter. On the other hand, if they originate from the nephrostomial canals, as appears more probable, the condition is akin to that described by Allen and Fraser. It differs from the latter author's chiefly in that the rete is formed in the gonad region from nephrostomial rudiments formed immediately dorso-lateral to it, and not from the region of the urogenital ridge anterior to the gonad. It is obvious that the rete tubules are primarily united with the mesonephric tubules, and that the union with the germinal elements is secondary. These findings are therefore distinctly contrary to those of Felix and Wilson.

The method of formation of the urogenital connection in the mouse, although simple in itself, is thus extremely difficult to interpret in the light of other mammals. This is due to the small size and rapid development of the gonads of the mouse and the vestigial nature of the Wolffian body.

In view of the fact that, in the mouse, the epithelial proliferation in the gonad is not, at first, in the form of cords, no conclusion can be drawn as to the homology of the nephrostomial canals and the " sex-cords " described in other forms. In the mouse the mesenchyme does not appear to contribute to the urogenital union itself, but its down-growth into the gonad results in the formation of the mediastinum and breaks the epithelial nucleus of the gonad up into spermatic or ovarian cords.

No connection was observed between the suprarenal primordia and the nephrostomes, such as has been described by Fraser and other authors. Indeed, as these, in the mouse, are widely separated by the gonad, the suprarenal *anlage* being median to it, and the nephrostomes lateral, no relation between them could exist.

The Müllerian duct develops, as stated by Felix (7) and de Winiwarter (12), in the human species, as a back-growth of the inner end of the ostium abdominale tubæ. The similarity in structure of the primordium of the ostium abdominale tubæ to the nephrostomes, and its similar position in the urogenital ridge slightly anterior to them, suggests that they may be homologous. On this view the ostium abdominale tubæ would be a nephrostome which grew and extended posteriorly to form the Müllerian duct.

5. *Summary.*

1. The differentiation of sex is accompanied from the beginning by an increase in diameter of the testes as compared to the ovaries. This can be detected by comparing the cross-sectional areas of the gonads of embryos from the same uterus at and after 11½ days *p.c.* It can be detected in micro-dissections at 12½ days *p.c.*

2. The mesonephros of the mouse is small and non-functional. It is composed of about ten mesonephric tubules. No glomeruli are formed, but rudimentary capsules of Bowman are represented by slight swellings on the distal ends of the tubules. About eight of the most anterior of these latter are connected with the peritoneal epithelium, immediately dorso-lateral to the gonads, by nephrostomial canals.

3. The urogenital connection is effected by the down-growth of strands of cells from the nephrostomial canals which fuse with the epithelial nucleus of the gonad and form the rete. The connection is established in the male at 12 days *p.c.*, and slightly later in the female.

4. The six to eight tubules united with the gonad by the rete cords form the epididymis or epoophoron. The blind posterior tubules form the paradidymis or paroophoron; they degenerate completely.

5. The ostium abdominale tubæ arises as an invagination of the peritoneal epithelium into the urogenital ridge anterior to the gonad and nephrostomes. It is suggested that it may be homologous to the mesonephrostomes.

6. The Müllerian duct is formed by the growth backwards of the inner end of the ostium abdominale tubæ.

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(11) de Winiwarter and Sainmont, 'Arch. de Biol.,' vol. 24 (1909).
(12) de Winiwarter, 'Arch. de Biol.,' vol. 25 (1910).

DESCRIPTION OF PLATES.

GUIDE LETTERS.

A., Aorta. A.m.s., A. mesenterica superior. A. umb., A. umbilica. B., Bladder. B.V., Blood vessel. C., Ovarian cortex. D., Diaphragm. E.T., Tubule of epididymis or epoophoron. G.C., Genital cord. G.R., Germinal ridge. G.T., Genital tubercle. K., Kidney. Lig. O., Ligamentum ovarii. Lig. T., Ligamentum testis. M., Medulla of ovary. M.C., Rudimentary capsule of Bowman. M.D., Müllerian duct. Mess. j., Mesonephric fold (tubal portion). M.T., Mesonephric tubules. N., Nephrostome. O., Ovary. O. abd., Ostium abdominale tubæ. Pl. d., Plica diaphragmatica. Pl. ing., Plica inguinalis. P.R., Pre-gonal region of urogenital fold. R., Rectum. R.t., Rete tubules. S., Ridge formed by the developing suprarenal and kidney. Supr., Suprarenal. S.T., Spermatic tubules. T., Testis. T.R., Tubuli recti of testis. Ur., Ureter. V.c.i., V. cava inferior. V.h.a., V. hemiazygos. W.D., Wolffian duct.

PLATE 13.

FIG. 1.—Dissection of 12½-day *p.c.* ♀ mouse embryo. The long narrow ovaries are seen attached to the small mesonephric fold. Medial to each ovary is a swelling in the body wall produced by the developing suprarenal bodies and the kidneys. The cephalic extremity of each mesonephric fold, showing the ostium abdominale tubæ, is attached to the diaphragm, which is cut across, by the short plica diaphragmatica. × 15¼.

FIG. 2.—Dissection of 12½-day *p.c.* ♂ mouse embryo. The testes are much thicker in proportion than the ovaries of the ♀ embryo from the same uterus (fig. 1). The loops of the spermatic tubules can be seen as slight ridges on the surface of the testis on the right-hand side of the picture. The ureters are seen as two ridges on the dorsal body wall, and the inguinal folds are clearly visible. × 22.

PLATE 14.

FIG. 1.—Dissection of 15-day *p.c.* ♀ mouse embryo. The ovaries are being pushed laterally by the developing kidneys, which are now distinguishable from the suprarenal bodies. The plica diaphragmatica is long. The ostium abdominale tubæ is clearly visible. The ligamentum *ovarii* and plica inguinalis are clearly shown. The junction of the tubal portions of the two mesonephric folds to form the genital cord is visible just in front of the bladder. In this specimen only one umbilical artery could be distinguished. × 13¾.

FIG. 2.—Dissection of 15-day *p.c.* ♂ mouse embryo. The testes are much larger and more ovoid than the ovaries of the female embryo from the same uterus (fig. 1). The loops of the spermatic cords can be seen as ridges on the surfaces of the testes. The testis on the right of the picture is approximately in its original position, but that on the left is undergoing a torsion about the mesonephric fold and is also being displaced laterally by the developing kidney. × 17½.

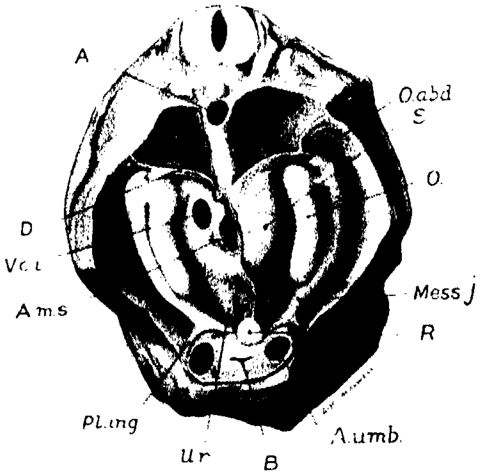

Fig. 1.

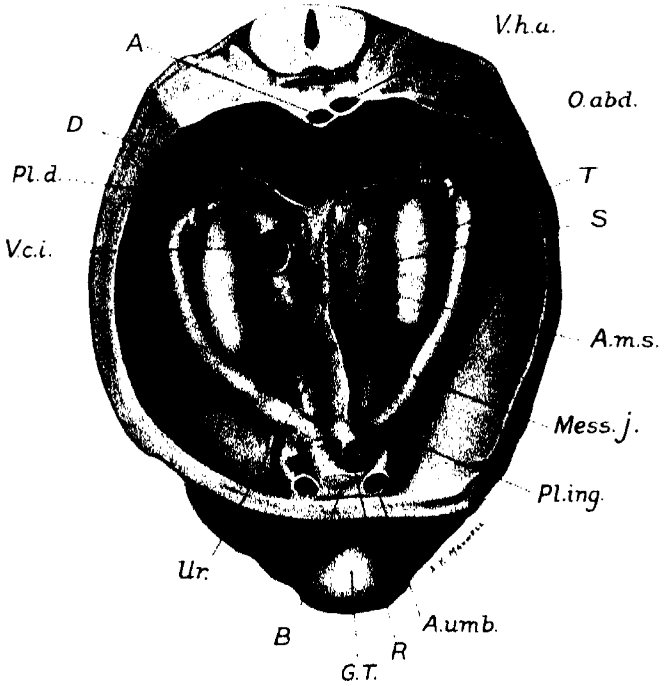

Fig. 2.

(*Facing p.* 220.)

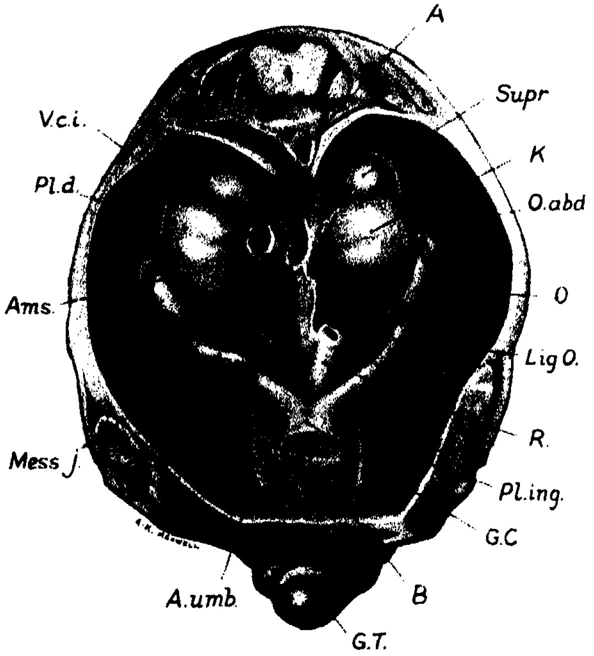
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FIG. 1.

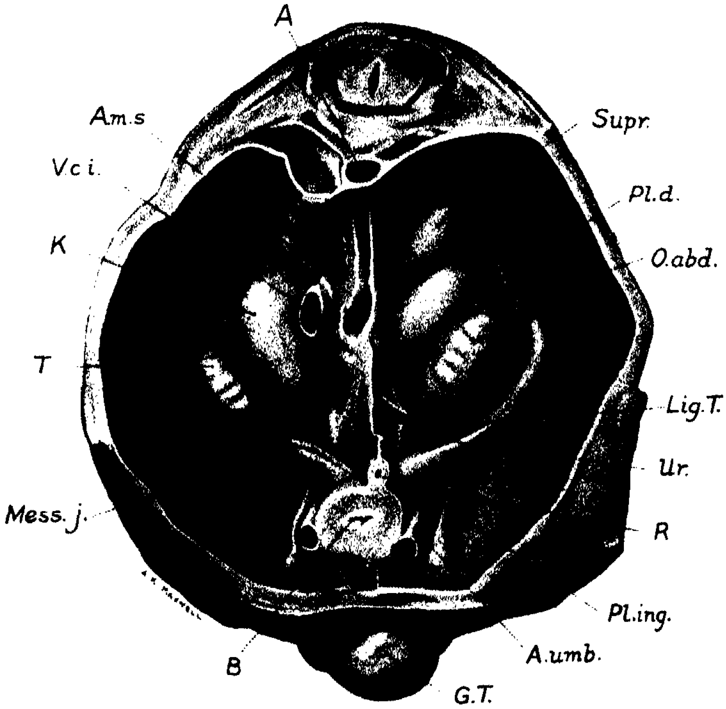
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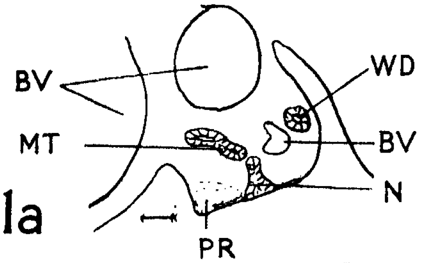
FIG. 2.



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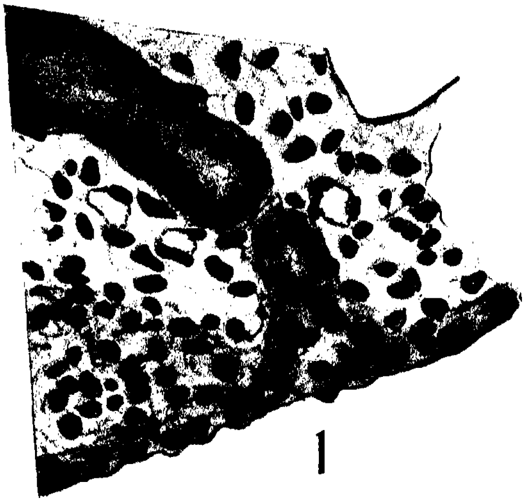

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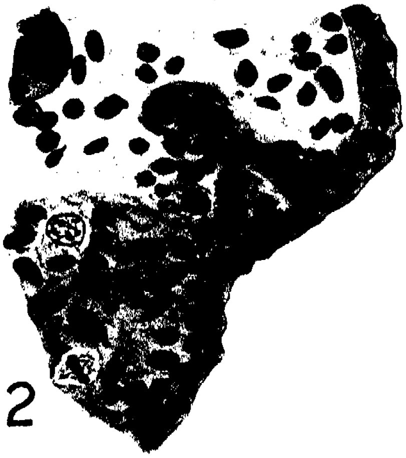

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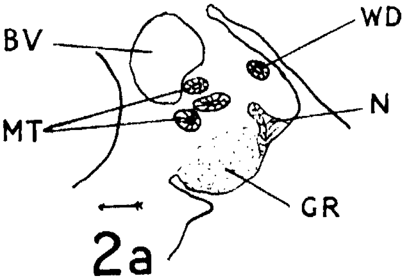

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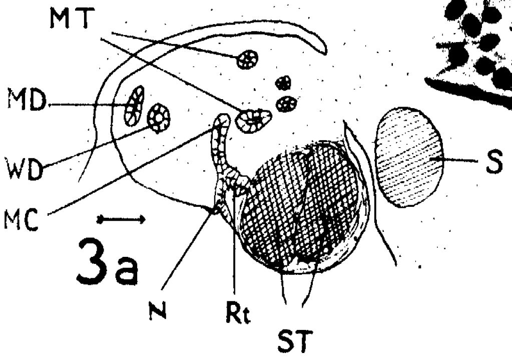
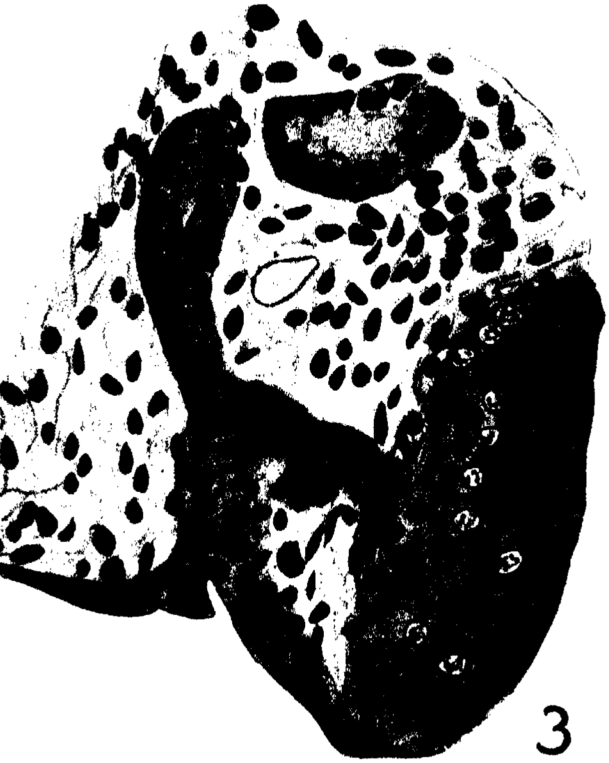


3a



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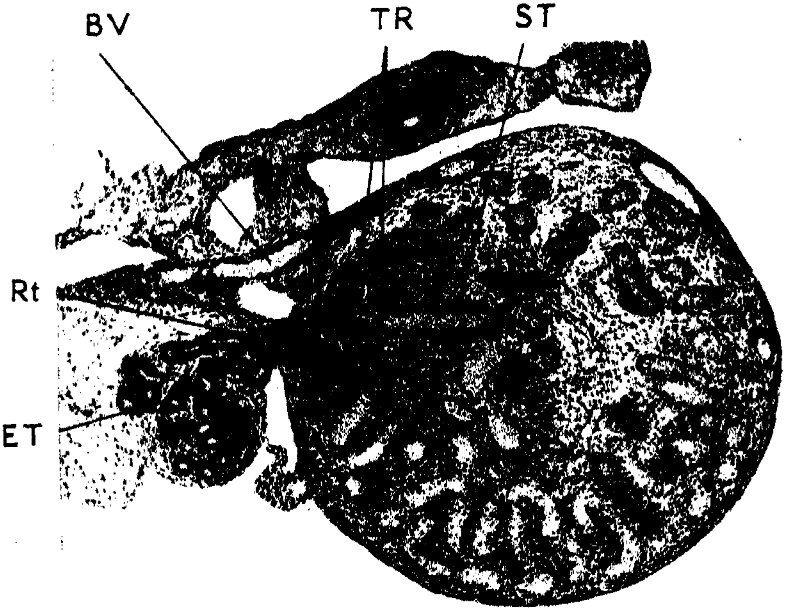

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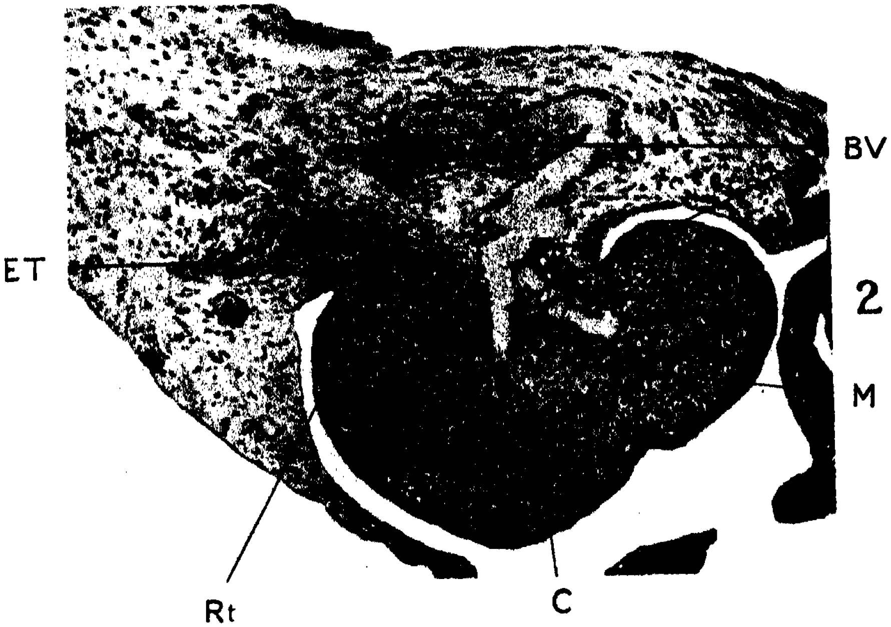

2

Responses to Stimulation of the Motor Area of the Cerebral Cortex.

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(Communicated by Sir Charles Sherrington, O.M., F.R.S.—Received October 1, 1927.)

(From the Physiological Laboratory, Oxford.)

[Plates 17 and 18.]

In a previous paper on concurrent mechanical and electrical responses to stimulation of the motor cortex in the monkey (3), we reported the transmission of stimulus rates of 18 to 68 a second. We were unable at that time to carry the enquiry farther owing to lack of means of obtaining higher variable rates of stimulus. Since then we have improvised apparatus giving higher rates and have been able to continue this part of our investigation, using rates up to 290 a second. The object was to determine the upper limit of rate of stimulus which would be transmitted directly to the muscle. We have also been able to collect much information as to the effects of low rates of stimulus, latent periods and the nature of cortical motor innervation as a whole.

In the course of the work six further monkeys were used, all small *Macacus Rhesus*. Of these two had also been used for experiments having no bearing on the present subject and not of a nature to affect our results. The animals were prepared as previously described and the muscles from which records were obtained were *M. brachialis anticus*, *M. flexor profundus digitorum*, *M. extensor carpi radialis* and *M. triceps brachii*.

The stimulation of the cortex was by means of a unipolar electrode with the indifferent electrode on the foot of the same side. Usually we made use of a fine stigmatic electrode; but a short, slightly curved strip of silver, 2·5 mm. wide, coated electrolytically with NaCl just before the experiment, was used as stimulating electrode a few times. The flat type of electrode was designed to sum the effects of a greater number of cortical points, but though occasionally giving good results it was found that the fine electrode produced better responses.

Several types of apparatus for producing rhythmic series of stimuli were used, a torsion wire key gave reliable series of break shocks from 35 to 90 a second; above that rate we used most often a rotating commutator with 10 brass sectors, and the spaces between filled with vulcanite, giving steady responses between

20 and 300 a second. In the latter apparatus the make shocks were not short-circuited, but they failed to stimulate except in a few instances which were easily detected, and our rate of stimulus was calculated from the rate of break shocks. A slow rotator gave us series of break shocks up to 15 a second. A coreless coil was used with each of these. An inductor alternator with no friction contacts was also employed for high rates of stimlus, but as a high potential variation was required to cause excitation and consequent escape of the stimulating current to the galvanometer record proved troublesome, the use of the machine was not continued.

The records were made as before by means of a torsion wire myograph with optical magnification. The galvanometer (Cambridge pattern, 1·3 μ silvered quartz string) recorded in the same optical system. The string tension was from 2 to 5 mm. per millivolt and the magnification × 390. The leads were of Ag. AgCl type in the form of thin pins inserted directly into the exposed muscle. The aponeuroses of extensor muscles gave us much trouble by facilitating escape of the stimulating current to the string, even after great care in cleaning fascia from the surface of the muscle.

Results.—Stimulation at High Rates.

For the results of very rapid rates of stimulation (120 to 290 a second) we draw mainly from two experiments. In the first of these the inductor alternator was used and only a few records show absence of escape of the stimulus itself. Our deductions were amply confirmed in the second experiment, where the rotating commutator was used, and many more reliable records were obtained. In the first experiment *Brachialis anticus* was used and in the second *Flexor digitorum profundus.*

The actual tensions developed in the mechanical responses have been small, only occasionally exceeding about 2,000 gms. and often much lower than this. We have no records of a maximal motor response, so that we are unable to say what fraction of the muscle is responding to cortical stimulation; but by comparison with the cat (4) the maximal response in one of the small wrist muscles is probably approaching 3,000 gms. and in *Brachialis anticus* it would be at least twice this tension, so that only a small fraction responds in most experiments. This is to be expected from the wide representation of each muscle on the cortex and the simple connection between each cortical point and the anterior horn cells. The electrical responses were never quite regular, though it was generally not difficult to recognise primary waves. In the first experiment, at a rate of stimulation of 90 a second from the torsion wire key, the primary waves follow each shock of the stimulus and there is usually found one secondary wave to each primary wave, thus making a total rate of 180 a second. Very occasionally there seem to be two secondary waves, so that for short intervals the rate is higher, but not much above 200 a second in periods of 1/10 second. When the alternator was used at rates of 200 and 220 a second, a few primary waves once followed the stimulus for a short interval, but were not repeated. From this experiment we concluded that the action currents would usually show a rate of about 180 a second and that rates close to this would be followed at a slightly higher rate, as was found in the flexion reflex in the cat (Cooper and Adrian (2)), when the higher limits were sought for.

By analogy with the cat one would expect this irregular rhythm of 180 a second to appear in a contraction not set up electrically, and in the course of this experiment we were able to get a record of a maintained autogenous flexor contraction in which the highest measurable rate is 175 a second and the average rate over longer periods is very little below this. It is of interest to mention at this point that in a maintained autogenous extensor contraction in the short head of triceps, the average rate is 140 a second; in places two stimuli are separated by as little as 0·0048 second (205 a second), but this was exceptional.

In the second experiment, where the rotating contact breaker provided the stimulus, the records are quite uncomplicated by escape, and a greater number of stimulating rates from the same apparatus were used, varying between 27 and 290 a second; these are best dealt with in order.

27 *a Second.*—The records show recruitment, and during the recruitment the stimulus rhythm is well marked in the electrical response; as the record proceeds and the plateau is reached, the secondary waves develop more and more and the primary get irregular in size and are not so marked, a total number of 180 to 200 a second is finally reached.

83 *a Second.*—A similar development of the secondary waves is seen during recruitment, the electrical response simply follows the stimulation rate in the early recruitment; then there is a very obvious doubling of the rate and occasionally a trebling, so that a rate of 166 shows clearly, and in places there are two secondary waves to each primary and the total rate is raised to about waves, 200 a second.

130 *a Second.*—At this rate the responses follow with irregular secondary waves, making a total of 160 to 180 a second.

160 *a Second.*—The responses follow clearly and fairly regularly (Plate 17, fig. 1).

182 *a Second.*—This is the interesting rate as it is the highest rate at which

the electrical responses follow the stimulus. (Fig. 2 shows short periods of regularity. This regularity by no means lasts throughout the record, and the rate may vary from about 160 to 190 a second, but in several places it is exactly 182 a second.)

200 *a Second.*—At this rate the responses definitely do not follow the stimulation rate, but a half rate is quite clearly seen and secondary waves make up the total rate to an average of 160 to 170 a second (fig. 3).

290 *a Second.*—The records at this rate are very similar to those at 200 a second, that is, they are irregularly 130 to 170 a second, with signs of half rhythm (fig. 4, 285 a second).

From these results it seems decided that the flexor muscles of the monkey can respond to rhythmical cortical stimulation by following up to something between 180 and 200 a second. At higher rates than this there is generally an irregular rhythm of about 180 a second, with a tendency to show a half rhythm, and at lower rates secondary waves are introduced irregularly to make up the total to about 180 a second. With rates of about 90 a second a regular double rhythm may show.

Stimulation at Low Rates.

In our previous paper we used rates of stimulation as low as 18 a second; in the present work we investigated the effects of rates down to 4 a second. At these low rates the mechanical record always showed a discrete wave in response to each stimulus, and recruitment was clearly marked. If the responses were recorded on fast plates an interesting picture of the electric responses was obtained. Fig. 5 was taken just at the end of recruitment at a stimulus rate of 12·5 a second, and a comparison of the first and fourth responses shows very clearly the development of eight or nine well-marked waves (see also Plate 18, fig. 6); the primary waves also develop in size progressively. The size and number of secondary waves and the size of the primary waves are in accordance with the size of the mechanical response, and this seems very much more noticeable at rates below about 20 a second; above this rate the size of the secondary waves more nearly approaches the size of the primary, and their number always tends to make the total number up to the limit, so that difficulty is found in picking out the stimulating rate at once.

At these low rates there seems to be a lower limit at which the cortex will respond; this limit in one monkey was about 8 a second, in another 4 a second, and lower rates gave no responses, even though the stimulation was continued for half a minute or more.

Facilitation and "Summation Period."

The time which elapses between the onset of the stimulus at the motor area and the first appearance of discharge from the final motoneurone we call the "*summation period*," to distinguish it from the latent period, which will be considered later. The summation period varies in length with the frequency of the break shocks in the stimulus, but remarkable constancy is found for any one rate of stimulus, provided that at least 30 seconds has elapsed since a previous observation.

If a stimulus be reapplied within 30 seconds after a previous stimulus, the summation period is markedly shortened, and this facilitation can be further enhanced by again ceasing and reapplying the stimulus. This reduction in summation period is often astonishing. When the rate of stimulus was slow, *e.g.*, 6 break shocks a second, the summation period was much prolonged, in some cases 20 to 30 seconds, but was shortened from 20 seconds to 0·18 second in one case by one facilitation.

By spacing observations at a minute apart, the facilitation of a response by the one before could be avoided, and it is in such a series that the relative constancy of duration of the summation period is a marked feature. Thus at a stimulus rate of 6 a second in one monkey, the summation period in four consecutive observations was 17, 18, 20, and 20 seconds (1 minute intervals); in another experiment, periods varying from 26 to 30 seconds were consistently found for a rate of stimulus of 5·7 a second. In one experiment we determined the summation period for a number of rates (photographic measurement), with each determination 1 minute interval after the previous observation (the degree of anæsthesia was very variable). It will be noted that the periods, which are given in Table I, are much shorter for given rates than in the two animals mentioned above.

Table I.

Rate of stimuli per second.	Summation period.
	Seconds.
13½	2·3, 2·1, 2·2.
13	2·3, 3·8, 4·0, 3·6, 3·2, 4·2, 6·5, 5·0.
10	5·8.
9	7·6, 9·6, 14·8, 6·8, 11·2, 14·0.
8½	10.
6	No response in 30 seconds.

It is conceivable that with a constant level of anæsthesia a definite mathematical relation between rate of stimulus and summation period could be obtained. We would draw attention, however, to the probability, for which reasons are adduced later in the paper, that this period represents not simple accumulation of excitation in the Betz cells, but the summation of excitation in the face of a rapidly developed inhibition in centres probably subcortical in situation.

Under approximately similar conditions of anæsthesia and with the same strength of stimulus we then determined the summation periods of the same animal after a subconvulsive dose of strychnine (0·13 mlg. per kilo) (Table II).

Table II.

Rate of stimuli per second.	Summation period.
	Seconds.
9	4·9, 4·3.
6	10·4.
4	No response.

Thus strychnine almost halves the summation period and also enables a response to summate when the rate of stimlus was too slow to cause any summation before the injection. The rate or amount of clonic after-discharge was not measurably increased.

Latent Period.

Distinct from the summation period is the true latent period of the impulse when once set up. During a response the time elapsing between the occurrence of a break shock at the motor area and the appearance of the first of the resulting volleys as an action current in the muscle fibre is measured with ease in records where the stimulus rate is slow, and the muscle action currents show a discrete wave or series of waves in response to each break shock.

Our measurements have been made from a minute notch in the galvanometer record, corresponding to "stimulus escape," to the beginning of the first resulting action current. In one animal the latent period averaged 16 σ; this is considerably shorter than the value of 33 σ found by Hoffmann (10) (dog). In another animal the average was 14 σ. In the latter experiment it was possible to measure the diminution in latent period of each impulse as the contraction gained in tension. Fig. 6 shows 14·2 σ, 14·2 σ, 13·4 σ, 13·2 σ, 13·2 σ and 14·0 σ as the latent periods of six consecutive volleys in one response. After a light dose of strychnine this was reduced to an average of 8·8 σ. The latent period of the muscle action current in response to a break-shock stimulus to the cut motor nerve in this animal under similar depth of anæsthesia was 2·5 σ.

These latent periods may be compared with the tendon jerk in the cat, which has a normal latency of ± 5 σ (Jolly (12), Denny-Brown and Liddell (5), Fulton (7)) and a motor nerve latency similar to the monkey.

We think, however, that it would be erroneous to argue from this evidence alone that the path taken by the impulse from the motor area to the muscle involves more synapses than a tendon jerk. And indeed, the transmission of high rates of stimulus is direct evidence that the contrary is the case. Denny-Brown and Liddell (5) have seen the latent period of the tendon jerk markedly prolonged by inhibition, and, as will be discussed later, there is evidence of inhibition having caused the prolongation of the latent period of an impulse from the cerebral cortex. The " latent period " measured by François-Frank and Pitres (6) is obviously the " summation time," and that these authors should find large differences in this period between the motor area and corona radiata is not surprising in view of the fact that even a slight change in the point stimulated in the motor area causes an alteration.

The General Nature of the Electric Response.

The action currents in these experiments follow the stimulus only for short series when the stimulus rate has been higher than 50 a second. In each series a succession of large " primary " waves occurs, one in response to each break shock at the cortex, and, depending on the rapidity of stimulus rate, secondary wavelets are interspersed with the primary waves to make up the total rhythm. That this process of " following " having once occurred does not go on for longer periods is surprising, and some pains have been taken to determine the character of the discharge which takes its place. In many cases the replacement is by an irregular succession of small secondary waves with here and there a large wave not related to the stimulus. In some records, however, a marked dominant rhythm of larger secondary waves makes its appearance, and a short description of secondary rhythms is first necessary to explain its significance.

In our preliminary paper it was pointed out that accentuation of each second or each fourth volley in the driven discharge at low rates was a common finding, so that the electrical and mechanical records at first sight have in places the appearance of being produced by a rate of stimulus one-half or one-fourth the actual rate used. This natural accentuation (instrumental causes being ruled out) is found in places in many of our subsequent records and, indeed, is also a common occurrence in reflex responses in the decerebrate or spinal cat. It was also seen that, following the appearance of a clonic rhythm in the mechanical response, the action currents took on a rhythmical waxing and waning of size (*i.e.*, height of deflection) corresponding to the beats of mechanical clonus, and we had instances when the two types of accentuation of action currents (halving and clonus) were superimposed.

Using higher rates of stimulus, clonus (2 to 10 a second) occasionally appeared in the mechanical response, and with it a corresponding rhythm of action current accentuation. Halving of the stimulus rate was also common, the interval being occupied by a small primary wave if the response were "following" the stimulus, or only by a few secondary waves (asynchronous with the stimulus) if it were not strictly following.

Between rhythms of accentuation which were quite slow, and therefore could be called clonus (2 to 10 a second), and "halving" of the stimulus many intermediate rates of accentuation occurred. These accentuations were often 25 to 50 a second, and their rhythm bore no relation to the stimulus rate which was visible all through the accentuation (figs. 1, 2, 3, 4 and 8). These natural rhythms were more or less constant in rate for several observations, but varied widely over a whole day's experiment. They are seen typically in the records, and parts of records, when the action currents do not follow the rate of stimulation.

This type of rhythm, more or less regular, neither following the stimulus rate nor being a submultiple of it and not of instrumental origin, began either by direct accentuation of primary waves (figs. 1 and 2) or by rhythmic accentuation of an irregular secondary discharge (figs. 3 and 4). The dominant rhythm resulting varied from 35 to 50 a second, and instances were seen especially often when very high rates of stimulus were used. We can advance no reason for its occurrence, but would venture to suggest that it bears a relation to that transient dominant rhythm of about 50 a second in the electromyogram of voluntary effort first observed by Piper (14).

Clonic After-Discharge.

Clonic after-discharge presents a number of different forms, each capable of transition into another during the outburst, and all types being found in the same preparation. No particular cause could be related to any one variety, and the only factor common to all forms seemed to be that the exciting stimulus was strong.

The most usual form of clonus is that of rhythmic beating from 2 to 5 a second (figs. 8 and 9) and more rarely as fast as 10 a second. Each beat of the clonus is an abrupt tetanus of asynchronous volleys at the rate of 160 to 180 a second, and consists of one or two small volleys, a large volley, and then one or two further small volleys, followed by a long silence until the onset of the next outburst (fig. 8).

The resulting mechanical record consists of a rapid rise of *d'emblée* type, a short plateau terminating often in a sharp " angle " followed by a rapid relaxation. The sharp angle is unquestionable evidence of practically simultaneous cessation of impulses reaching the muscle (Fulton (7)). This, together with the sharp relaxation and the silence of the string, strongly suggests the presence of inhibition lasting until the onset of the next beat. The short tetanus often shows two or three volleys separated by 1/25 to 1/50 second and larger than the others, and here the resulting mechanical record shows the fused twitches superimposed as in fig. 8.

By a slowly developing continuous discharge after every second beat, a simple clonus often passes gradually into one of half the rate (fig. 10), in which each beat now becomes a longer plateau. Or again, the latter variety may begin the after-discharge and pass gradually into simple peaked clonus of twice the rate. Rarely the whole discharge becomes a convulsant fit, where the troughs of the clonic beats are all partially filled in by a residuum of contraction, the whole disturbance showing the march of clonic epilepsy.

Clonic after-discharge does not, in our experience, begin by a beat superimposed upon the contraction response, but is invariably ushered in by a relaxation, in the majority of cases a complete relaxation, of the main response. In this respect it closely resembles the primary inhibition which is the first sign of the phasic scratch-reflex breaking into a posture under anæsthesia (Graham Brown (8)). The relaxation of the main response much resembles the relaxation of a beat of clonus, and sometimes the tension of the clonic beats is exactly the same as that of the response itself, never higher, but usually lower. From this it can be deduced that the number of motor units taking part in clonic after-discharge is the same or fewer than those taking part in the direct excitatory response. It is considered possible that they are the same motoneurones.

On a few occasions when we were recording from both flexor and extensor, concurrent excitations occurred in both antagonists. In most cases reciprocal innervation could be seen to take effect when the combined tensions of the antagonists reached values which would indicate stress in the intact limb. Concurrent clonus occurring reciprocally in extensor and flexor has been observed by previous investigators (Graham Brown and Sherrington (9)), and we have been able to record examples of it. We have one example (fig. 11) where the onset of each clonic beat was simultaneous in both flexor and extensor, but it will be observed that the beats, which are multiple, show a reciprocal relation in their further course. The flexor beat shows a second discharge raising its tension while the extensor merely maintains its tension or shows a fall.

Concurrent contraction plateaux in two antagonists may indicate that fixation of that joint is occurring and the primary movement is elsewhere. As our muscles are isolated by paralysis of the remainder of the muscles in the limb, we have no evidence to bring forward to support this. It is considered more likely, however, that although a reciprocal relationship attends each path from the cortex, the usual motor response elicited by a stimulus applied to the surface of the motor area is but an algebraical sum of the varying elements excited—a double reciprocal innervation from the excitation of antagonistic units.

Discussion.

We find the upper limit of the rate at which the spinal discharge will follow rhythmic stimulation of the cortex to be about 180 a second. No evidence is found for the 10 per second rhythm which Horsley and Schafer (11) and Richet (15) considered to limit the discharge of the motor cortex nor of the 50 per second limit which Hoffmann finds for the dog (10), although superimposed rhythms of these rates have occasionally appeared. Our results would suggest that the limiting factor is of the type found in the flexion reflex in the spinal cat, where probably but one synapse is involved. That collaterals from the pyramidal tract in the monkey ramify among the anterior horn cells was described by Leyton and Sherrington (13), and a direct synaptic relation seems most likely.

The general type of response has consistently remained that which was described in our previous note, namely, a recruiting ascent with an abrupt termination upon cessation of the stimulus. The sloping ascent is clearly due to the gradual involvement of more and more units as the contraction develops; it is a true recruitment, not a mere increase in the rate of discharge. In this respect and in the gradual development of secondary waves tending to smother the driving rhythm, cortical movement has much in common with the reflex of crossed extension, and is the expression of excitation developing against resistance. While crossed extension has a resistance attributed to multiple synapses, another explanation would seem to hold for the cortex.

The termination of the cortical response is characteristic and the rapid cessation of discharge and the abrupt fall in tension have been the rule for both flexors and extensors in our results. The exception to this type of termination is rare and is seen when the original response has been a powerful irregular clonic outburst continuing when the stimulus ceases. In this case the relaxations from the clonic beats at once deepen with the cessation of the stimulus, and a suggestion is given of a regular plateau underlying the original disturbance, partially filling up the troughs of the clonus and vanishing with the cessation of the stimulus.

The inevitable relaxation which characterises the onset of clonic after-discharge in our records suggests that the *relaxation* of the main response *per se* is the exciting cause of clonic after-discharge. That this is not the case, however, is indicated by its occurrence in the deafferented muscle. We have had the opportunity of recording the responses to cortical stimulation in monkeys with a deafferented fore-limb, in the course of experiments with Sir Charles Sherrington, to be published later. Clonic after-discharge and its onset retained all the characteristics we have described in the intact animal.

If the clonus is not peripheral in origin, then it may be profitable to consider its resemblance to phases of inhibition followed by rebound. The abrupt relaxations and the silence of the string both strongly suggest inhibition as a cause, and it is well known that rhythmic discharges elsewhere can be provoked by a conflict of inhibition and excitation.

In a few instances where there has been an initial posture at the time of application of the stimulus, an inhibition with a relatively short summation period has occurred (fig. 7) and lasted obviously up to the beginning of excitation. That this inhibition should last all through the response is not impossible, and is perhaps evidenced by the decreasing latent period of each impulse. Thus the long summation period, the recruitment, the abrupt termination, and the rebound-like appearance of the first beat of clonus would simply be the expression of the excitation breaking through a background of inhibition, while the accumulated excitation later reasserts itself as rhythmic clonus. Rebound as explanation of clonus is further supported by an observation of Graham Brown and Sherrington (9) that "epileptoid discharge in the flexor muscle following on stimulation of a flexor cortical point is increased by subsequent stimulation of an extensor cortical point, while restimulation of the flexion cortical point diminished the epileptoid discharge during the period of the stimulation."

One or two responses without clonus which have shown a momentary terminal rebound (figs. 1 and 11) strengthen this view, as this phenomenon in decerebrate reflexes is well known to indicate a concurrent inhibition. As there is usually no initial posture, owing to the depth of anaesthesia used, there is no initial indication of inhibition, but its presence during all recruitments in these responses is not improbable. Only one of our records shows maintained discharge following the cessation of the stimulus and a resulting mechanical response, which remains steady at about one-third plateau tension. We suppose that this represents the "tonic" after-discharge described and figured by earlier workers (6, 9 and 11), and we consider it evidence of an uninhibited fraction of the activated units.

François-Frank and Pitres (6) obtained epileptiform after-discharge unaffected by removal of the cortical centres, and we regard this, as well as the close resemblance of clonic after-discharge to the well-known clonus in spinal mammalian preparation, as true indications that clonus and also probably faster dominant rhythms are features whose seat of origin is the spinal grey matter.

All the evidence is suggestive that the whole form of the response to stimulation of the motor cortex, and the clonic after-discharge characteristic of that response, are the outward signs of a conflict of summated excitation and inhibition. This conflict, which is not obviously a feature of normal voluntary movement, is not unlikely the result of artificial provocation to concurrent activity of two naturally opposed groups of minute cells in the mosaic of the motor area. One of these groups produces excitation at the final motor neurone and the other inhibition. The inhibition so rapidly developed is not merely the expression of activity of the antagonist, indeed our double records show the resulting impedance in both antagonists at the same time. The inference from this we think is clear, that for a given movement the excitatory units and the inhibitory units lie microscopically intermingled in the cortex, and, further, that representatives of movements in opposite directions are often closely enough related anatomically to be excited by the same stimulus.

Conclusions.

1. The spinal discharge evoked by cortical stimulation can follow the rate of repetition of break shocks in that stimulus up to about 180 a second, indicating a very simple synaptic relation between the pyramidal tract and the anterior horn cell.

2. Rates of stimulus above and below that rate usually cause a total electromyographic rhythm of 160 to 180 a second.

3. Varying types of grouping of the spinal discharge occur, among them being that of a "dominant" rhythm of 35 to 50 a second, with no relationship to the rate of stimulus.

4. The power of summation of the cortico-spinal path is well illustrated by the relative constancy of the summation period. The comparative stability

of that period gives it especial value in investigating some aspects of cortical excitability.

5. The typical motor response to stimulation of the motor area shows signs of concurrent inhibition. Reasons are given for believing that clonic after-discharge, "epilepsy," and the forms of the motor response are the result of a conflict between inhibition and excitation.

We wish to express our thanks to the Medical Research Council for defraying the cost of six monkeys, and to the Christopher Welch Trustees for a grant towards photographic material.

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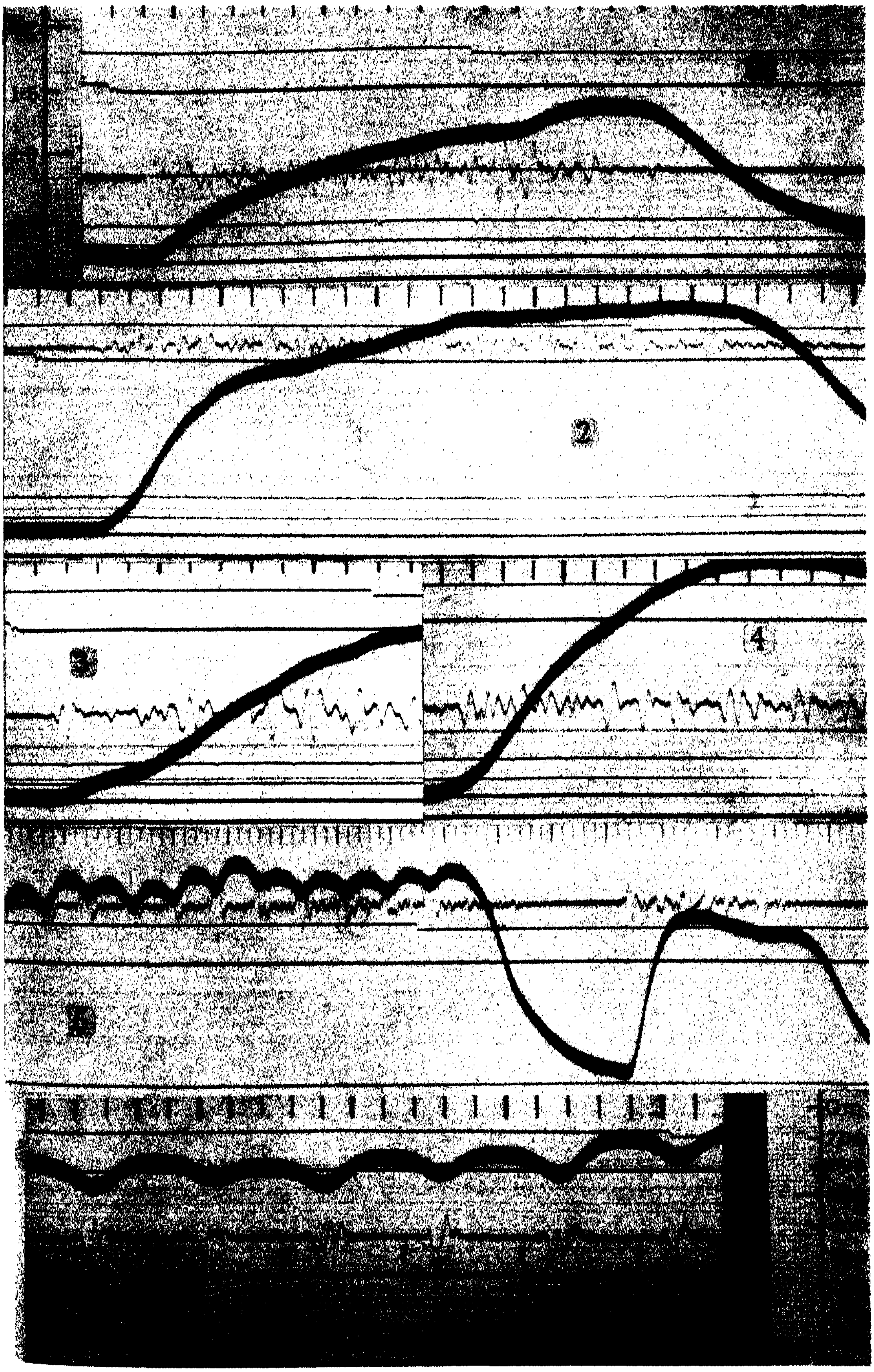
DESCRIPTION OF PLATES.

All figures read from left to right, and in all except fig. 11 an upward movement of the myograph line (thick) means an increase in tension. The tension scale for figs. 1 and 3 is indicated on fig. 1 and the scale for figs. 2, 4, 5, 7, 8, 9 and 10 is given on fig. 7. Figs. 6 and 11 have their own scales. The signals for "on" and "off" for the stimulus appear as rapid downward displacements of horizontal lines (photographic registration of movement of un-shortcircuiting and re-shortcircuiting levers). Time is shown by vertical strokes at the top of each record (Rayleigh wheel), and the intervals for each figure are given in its description.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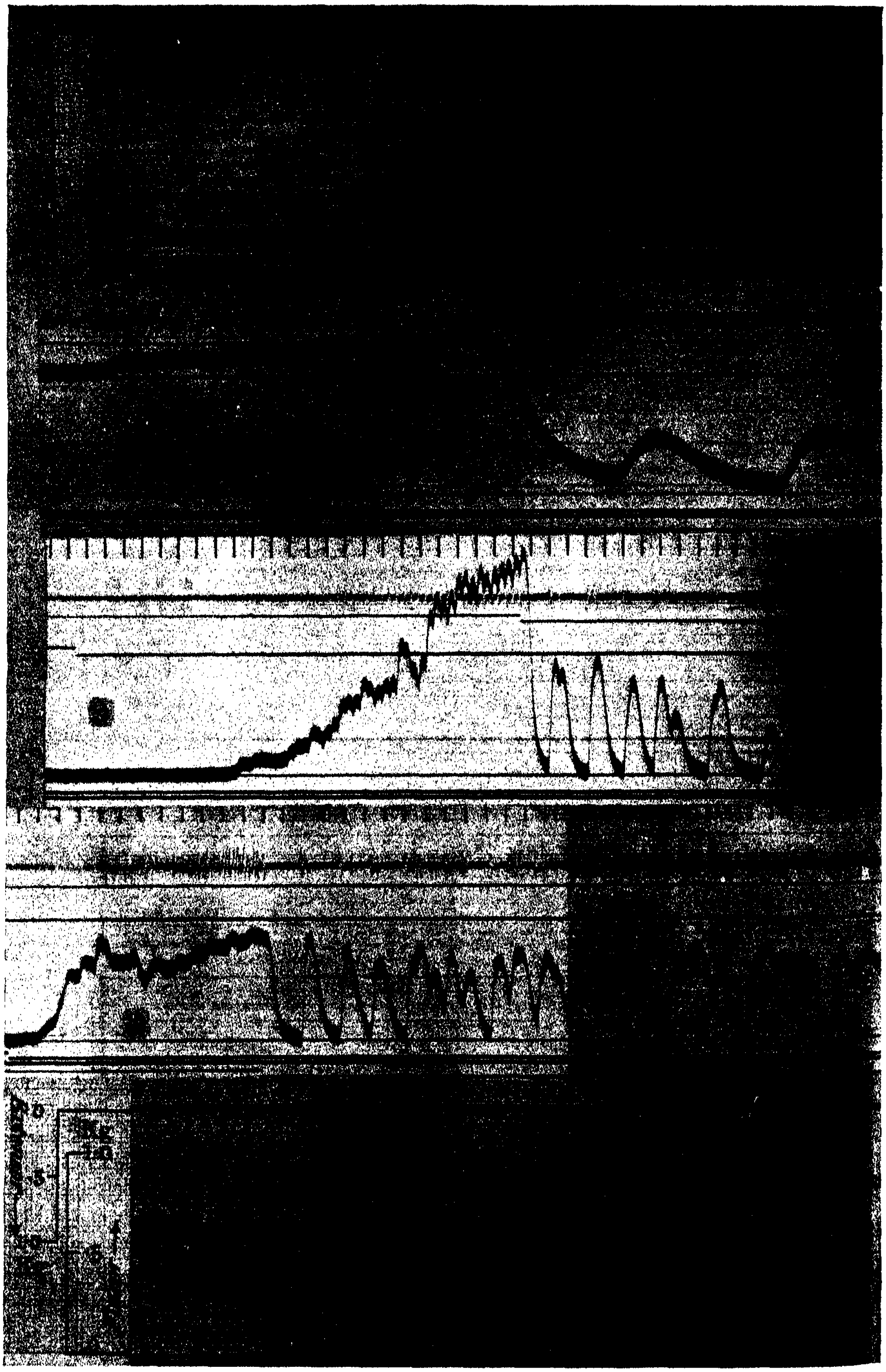
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PLATE 17.

FIG. 1.—Flexor Profundus Digitorum, stimulus rate 160 a second (notches in third line from bottom indicate every tenth stimulus) from rotating commutator. Time strokes 1/50 second intervals.

Note.—Electrical responses follow stimulus rate. Very few secondary waves. Accentuation of primary waves to produce a dominant rhythm of about 50 a second. Terminal rebound after cessation of stimulus.

FIG. 2.—Same muscle and conditions as fig. 1 except rate of stimulus now 182 a second. Time marker 1/50 second.

Note.—Electrical responses follow stimulus rate. Lack of secondary waves. Grouped accentuation at about 35 a second.

FIG. 3.—Same muscle and conditions as figs. 1 and 2 except that rate of stimulus is now 200 a second. Time marker 1/50 second intervals.

Note.—Lack of following of stimulus rate and tendency to show a natural "dominant" rhythm of about 40 a second, and a corresponding mechanical rhythm of about 20 a second.

FIG. 4.—Same muscle as figs. 1, 2 and 3. Rate of stimulus 285 a second. Time marker 1/50 second intervals.

Note.—Lack of following and natural rhythm of 25 a second appearing.

FIG. 5.—Flexor Profundus Digitorum of another monkey. Stimulus at 12·5 a second from a rotating contact breaker. The beginning of the stimulus was about 1 second earlier. Time marker 1/50 second intervals.

Note.—Large primary waves and gradual development of secondary waves, as the plateau is reached. Very little after-discharge and almost complete relaxation before the first beat of clonus, which in this case is a long slow type.

FIG. 6.—Same muscle and animal as figs. 1, 2, 3 and 4. Stimulus 13 a second from rotating contact breaker. Time marker 1/50 second intervals.

Note.—Secondary waves in response to each break shock. A minute upward deflection of the galvanometer string (indicated by arrow) shows instant of application of stimulus. True latent periods measured to onset of electrical response.

PLATE 18.

FIG. 7.—Same muscle and animal as fig. 5. Stimulus 50 a second by torsion key. Time intervals 1/5 second indicated by vertical strokes.

Note.—Preliminary posture, with accompanying action currents in the string, which is inhibited about 1/5 second after the onset of the stimulus. Slow recruitment, lack of after-discharge, and rapid relaxation, with clonus at about 6 a second.

FIG. 8.—Same muscle and animal as fig. 7. More rapidly falling plate. Time marks at 1/50 second intervals. Stimulus rate 50 per second by torsion key.

Note.—Secondary waves making up the rate to 130 a second. Grouping of accentuation at about 35 a second. Typical clonic after-discharge showing the short tetanic electric response with silence between beats and angle in mechanical response.

Fig. 9.—Same muscle and animal as fig. 8. Slow stimulus by rotating contact breaker at 12·5 a second. Time intervals 1/5 second.

Note.—Long summation period. Mechanical rhythm of stimulus in the slow recruitment and typical lack of plateau after-discharge, rapid relaxation and the usual type of clonic after-discharge.

Fig. 10.—Same muscle and animal as fig. 9. Stimulus 50 a second by torsion key. Time intervals 1/5 second.

Note.—Clonic after-discharge at 2·5 a second, gradually becoming 1·25 a second.

Fig. 11.—Lower myograph (pulling upwards) is of Brachialis Anticus. Upper myograph is Triceps (pulling downwards). Stimulus from torsion wire key at 50 a second. The cessation of the stimulus is marked by a signal near the lower left-hand corner of the figure. Time-marker strokes 1/10 second intervals.

Note.—Onset of concurrent clonus in the flexor and extensor, with the beginning of each multiple beat practically simultaneous in each. Note reciprocal relation of subsequent volleys in each beat. The extensor shows slight terminal rebound.

Linkage Groups and Chromosome Number in Lathyrus.

By R. C. Punnett, F.R.S.

(Received October 4, 1927.)

After the discovery of linkage of characters in the sweet pea, the question naturally arose as to a possible connection between these linked groups and the chromosomes. It was realised that a definite contribution would be made to such an inquiry if it could be decided whether the groups showing linkage, together with characters exhibiting independent assortment, were more numerous than the haploid number of the chromosomes, or not. With the advent of several fresh and easily worked characters, a definite series of experiments with this object in view was started in 1908, and since that time the work has gradually been extended as further favourable characters were met with.* While it was in progress the question at issue was definitely decided in the case of the little fruit fly, *Drosophila melanogaster*. The rapidity and ease with which this material could be bred placed it beyond a doubt that the number of linkage groups is here the same as the haploid number of the chromosomes, viz., 4. Hitherto, however, *Drosophila* has in this respect remained unique.

Of recent years a considerable amount of work has been done in connection with linkage groups in plants, notably in *Pisum*, *Antirrhinum*, maize, wheat and barley, but in no case is progress yet sufficient to allow of a categorical statement; and, indeed, there would appear to be an inclination on the part of some workers, notably among those with *Pisum*, to recognise a number of linkage groups and independently assorting characters greater than the haploid number of the chromosomes. For *Lathyrus*, however, the first species in which investigations of this nature were planned and begun, a definite answer has at last been obtained.

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* For some account of the work up to 1922, see 'Journ. Gen.,' vol. 13, pp. 101–123 (1923).

During the 20 years over which the work has extended, 19 characters have been made use of, and with relatively few exceptions each of these characters has been crossed with every other one. In many cases also both types of mating—viz., the **AB** × **ab** type as well as the **Ab** × **aB** type—have been made, especially in cases where the F_2 distribution was such as to make the existence of linkage appear doubtful. There are, of course, more than 19 characters in the sweet pea which are known to exhibit ordinary Mendelian segregation, but many of these, such as the finer shades of flower colour and the tint of the plastids, are unsuitable for work in this connection. Only such characters were used as were, in their various combinations, readily distinguished in the field. These characters, of which about one-half were structural, were as follows:—

A_1	Purple—red* flower colour.
A_2	Long—round pollen.
A_3	Erect—hood flower shape.
B_1	Dark—light axil.
B_2	Fertile—sterile anthers.
B_3	Normal—cretin flower.
D_1	Tendril—acacia leaf.
D_2	Bright—dull flower colour.
D_3	Self—flake flower pattern.
D_4	Hairy—glabrous.
D_5	Deep—picotee flower shade.
E	Tall—dwarf (cupid).
F_1	Colour—R-white.
F_2	Procumbent—erect (bush) habit.
F_3	Self—marbled flower pattern.
G_1	Colour—C-white.
G_2	Purple—copper flower colour.
G_3	Full—dilute (mauve) flower shade.
H	Clamped—open (Spencer) keel.

* The dominant member of the pair is given first throughout.

In the above list* the various characters have been denoted by one or other of the seven letters A, B, D, E, F, G, H. The characters grouped under a given letter are characters which exhibit linkage towards one another—*e.g.*, the three pairs dark—light axil (B_1), fertile—sterile anthers (B_2), and normal—cretin flower colour (B_3), all show linkage with one another and are presumably located in the same chromosome. On the other hand, the members of groups denoted by different letters have now been definitely proved to show independent assortment towards one another, and must therefore be supposed to be located in different chromosomes.

As the list indicates, there are five linkage groups, of which one (D) contains five members, while the other four (A, B, F, G) contain three members each. Besides these five groups there are two characters (E and H) which have hitherto shown no linkage, either with one another or with any other of the characters investigated. Hence the number of the linkage groups together with the freely assorting single characters is seven, which is the same as the haploid number of chromosomes. We have now a plant as well as an animal in which this fundamental postulate of the chromosome theory is fulfilled.

In the present note a brief outline only of the case has been given, leaving the detailed evidence for later publication, when the strengths of certain linkages have been determined by experiments now in progress.

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* A description of the various characters in the above list will be found in 'Bibliograph. Genet.,' vol. 1, pp. 69–73 (1925).

Address of the President, Sir Ernest Rutherford, O.M., at the Anniversary Meeting, November 30, 1927.

At this Anniversary Meeting we are naturally conscious of the losses suffered by our Society during the year. These include thirteen of our Fellows and three Foreign Members. We have also to record the loss of one of our Fellows under Statute 12, EDWARD CECIL GUINNESS, EARL OF IVEAGH, elected 1906.

Sir WILLIAM AUGUSTUS TILDEN passed away on December 11, 1926, in his 85th year. He was appointed Professor of Chemistry and Metallurgy in the Mason College, Birmingham, in 1880, and in 1894 became Professor of Chemistry in the Royal College of Science; he retained this latter position until his retirement in 1909. Much of Tilden's early work was concerned with the terpenes, and he was the first to show that the hydrocarbon, isoprene, undergoes polymerisation with formation of caoutchouc. His Bakerian Lecture in 1900 dealt with the relation between atomic weight and specific heat, and he was awarded the Davy Medal in 1908. A man of many accomplishments and a courtly gentleman, he gained the affection and esteem of all with whom he came in contact.

J. J. LISTER made his mark in the science of Zoology by his important discoveries on the life-history and morphology of the Foraminifera and by his contributions to our knowledge of Mycetozoa. During his travels in the Pacific Ocean he made many valuable additions to our knowledge in several branches of the science and enriched our collections of the organisms that construct coral reefs. His father and grandfather were Fellows of the Royal Society, and his uncle, Lord Lister, was formerly our President.

Sir GEORGE GREENHILL, for more than 30 years professor at the Royal Artillery College, Woolwich, was a mathematician of distinction and one of the foremost authorities on the science of ballistics. He especially excelled in the application of dynamics to the practical problems of everyday life, where his interests ranged from aeroplanes and ships to spinning-tops.

ARTHUR WILLIAM CROSSLEY was taken from us at the early age of 58 years. He was successively Lecturer in Chemistry in the Medical School of St. Thomas' Hospital, and Professor of Chemistry to the Pharmaceutical Society and to

King's College, London; as an accomplished organic chemist he contributed much to our knowledge of the hydro-aromatic compounds. On the outbreak of war, Crossley threw himself whole-heartedly into the national effort and became the first Secretary to the Advisory Committee of the Trench Warfare Department; he was given the rank of lieutenant-colonel, R.E., and undertook the organisation of the newly founded Experimental Station at Porton, a task which he performed with conspicuous success. After the war he became the first Director of the Shirley Institute, Manchester, the home of the research association dealing with the cotton industry. The results of Crossley's marked ability as an initiator of organised scientific effort will long remain as a memorial to a brilliant leader.

ERNEST HENRY STARLING, who died at the relatively early age of 63 years, was the first of the Royal Society's Research Professors, being appointed first Foulerton Professor in 1922. For more than 30 years Starling has been recognised as an outstanding figure in Physiology, and the investigations which he carried out with energy and enthusiasm have effected a clarification of knowledge and a new orientation of ideas concerning a succession of the most important functions of the body. His earliest studies, made like so many later ones in co-operation with his great friend and scientific partner, the late Sir William Bayliss, were directed to analysis of the action of the mammalian heart.

Though his activities were for many years diverted to other problems, his interest in the heart and the factors regulating its function remained with him always, and became again effective in his later years in a series of masterly researches. These he carried out, with a succession of able colleagues and pupils, on the mammalian heart, isolated by a method of his own devising, from the vessels of the major circulation, and performing its function under conditions which allowed the rate at which the blood entered the heart, and the resistance encountered in its ejection, to be artificially controlled—the well-known "heart-lung preparation."

In the intervening years he had completed and published, largely with Bayliss, a series of researches in which the physical laws governing filtration, diffusion and osmosis through membranes were applied to explain the formation and absorption of lymph. There followed further co-operative investigations with Bayliss on the movements of the intestinal walls, their co-ordination by the local nervous plexuses and their general control by the central nervous system, and then—most famous, probably, of all their joint researches—their work on the stimulation of pancreatic secretion following the discharge of the stomach contents into the small intestine. This led to the discovery of "secretin" and the formation of general conceptions of the control of bodily functions by chemical messengers or "hormones," embodied in Bayliss and Starling's joint Croonian Lecture before this Society.

During the war and the years immediately following it, Starling gave his whole energies to the service of the State, as director of investigations on defence against chemical warfare, as chemical adviser to the army at Salonica, and later as chairman of the Royal Society's Food Committee, Scientific Adviser to the Ministry of Food and British Scientific Delegate to the Interallied Food Commission.

When his thoughts and activities were at last free to return to their own natural channels, he began a large and exacting series of investigations in which he combined some of his earlier methods to study the formation of urine under conditions artificially controlled and varied. These researches with many which grew from them, he continued to prosecute during his tenure of the Foulerton Professorship. He enjoyed the assistance and collaboration of a succession of able colleagues and of visiting workers from many countries, eager to learn his methods and to imbibe his ideas. It might have been hoped that the new conditions of freedom from the responsibilities of administration and formal teaching would have enabled him to conserve his physical powers; but Starling's restless energy and burning enthusiasm forbade all thought of relaxation, till at length a physique, weakened by illness, broke under the strain.

By Starling's death the Royal Society has lost not only a great investigator who has rendered one of its Chairs illustrious by his brilliant record of research as Foulerton Professor, but a Fellow who has been generous of efficient service to the Society on its Council and its Committees, who has been proud of its traditions and jealous for its fame and prosperity. He was awarded a Royal Medal in 1913. In and beyond this Society he was a stalwart champion of the claims of research and of the interests of his scientific colleagues. Physiology, above all, was the central interest and enthusiasm of his life. In its faithful and single-minded pursuit he had always one end in view; this, to quote from his own beautiful Harveian Oration was "to attain to a comprehension of the 'wisdom of the body and the understanding of the heart,' and thereby to the mastery of disease and pain, which will enable us to relieve the burden of mankind."

By the death of GÖSTA MITTAG-LEFFLER, mathematical science lost one of its outstanding figures, one who played a notable part in the great development of the Theory of Functions which occurred in the latter half of the last century. Even more memorable, perhaps, than his additions to the growth of knowledge, was his foundation of the *Acta Mathematica* in 1882, a journal which he conducted with loving care for over 40 years to the inestimable benefit of mathematical science. He will be long remembered for his critical studies in the history of mathematics, for his activities at international gatherings, and for his outstanding personality.

HENRY RICHARDSON PROCTER, who died in his 80th year, was head of the Procter International Research Laboratory of the University of Leeds. His laboratory, as its name implies, was built by subscriptions from all over the world, to further by fundamental scientific research the advance of leather technology, and to commemorate Prof. Procter's unique services in the leather industry.

Prof. W. BURNSIDE was Professor of Mathematics at the Royal Naval College for over 30 years. Of much originality and power as a mathematician and distinguished as a teacher, his contributions to mathematical knowledge covered a wide variety of subjects, his earlier papers dealing mainly with hydrodynamics and waves, a large number of later papers with the Theory of Functions, and his more mature work with the Theory of Groups, a subject with which his name will always be associated. His book *The Theory of Groups*, first published in 1897 and again in 1909, is a standard exposition of the subject, much of it the result of his own researches.

Sir ARTHUR SHIPLEY was in his early years a well-known contributor to the scientific journals on Parasitology, but when appointed to the Mastership of a Cambridge College and involved in the administrative duties of the University, he devoted his great organising powers to the wider dissemination of scientific truths and to the application of biological knowledge to the solution of problems in Agriculture and Fisheries.

As a scientific adviser to several Government Departments, as Chairman of the Marine Biological Association, and in many other activities, he rendered most valuable assistance to the promotion of Science and the welfare of the community.

The death of Prof. A. LIVERSIDGE, in his 80th year, removes one of the pioneers of scientific education in one of our great Dominions. At one time Demonstrator of Chemistry in Cambridge, he was elected in 1873 to the Chair of Chemistry in the University of Sydney, and held this post for 35 years. He was active in promoting scientific and technical education in Australia and was largely responsible for founding the Australasian Association for the Advancement of

Science, which has been of signal service in bringing together the widely scattered scientific workers. An ardent collector of minerals and meteorites, his scientific contributions were mainly connected with experimental mineralogy. On his retirement he resided in England, where he had a wide circle of friends.

WILLIAM EINTHOVEN, for 42 years Professor of Physiology in the University of Leyden, Nobel Laureate in Medicine, was elected a Foreign Member of the Society in 1926. His long scientific career was devoted to the invention and perfecting of physical apparatus of the greatest delicacy and precision for the record and analysis of processes occurring in the living body or having a physiological interest. His string galvanometer, originally devised for recording the fleeting electrical changes which accompany the different stages of the beat of the heart, and now in world-wide use in hospitals as well as physiological laboratories, has also found application in a wide range of purely physical investigations.

The scientific world deplores the loss at the age of 67 of SVANTE ARRHENIUS, Foreign Member and Davy Medallist of our Society and Nobel Laureate. Shortly after his graduation he published, in 1889, a famous paper in which he advanced the theory of ionic dissociation to explain the properties of electrolytes. This theory, and the modifications of it proposed from time to time, have stimulated an enormous amount of research in all lands, and indeed Arrhenius may justly be regarded as one of the founders of modern Physical Chemistry. A man of wide scientific interest and almost encyclopædic knowledge, he made valuable contributions in many branches of science. As Director of the Nobel Institute in Stockholm he guided an active school of research, not only in problems of physical chemistry but in the application of physico-chemical ideas to physiological and biological problems, particularly to serum-therapy. Nor must we omit an excursion into the realm of cosmical physics, where he brought his wide knowledge to bear in interesting speculations on many subjects, such as the cause of variations of the earth's climate, the origin of our solar system and the dissemination of life throughout the universe. A man of forceful personality, of courageous and original mind, his early death will be mourned by a wide circle of friends throughout the scientific world.

HENRY MARTYN TAYLOR was known to many generations of mathematicians as Tutor and Lecturer of Trinity College, Cambridge. Failing eyesight restricted his activities in middle age, and after his sight was completely lost he devoted himself with energy to the preparation and publication of embossed books for the blind. Courageous in adversity, he continued to take an active part in public affairs and was Mayor of Cambridge and for many years a Magistrate.

In these notices I should like to include the name of A. A. Lawson, at one time Lecturer in the University of Glasgow and later Professor of Botany in the University of Sydney, who was a Selected Candidate for our Fellowship, but died before the date of formal election. His tenure of the professorship in Sydney was marked by the erection of a new Botanical Institute, which was opened in 1926 shortly before his death. A botanist of distinction and a worker in many lands, he made contributions to our knowledge in cytology and the morphology of the gymnosperms, and developed in Sydney an active school of research.

Turning to other matters, I would like to say a few words on some events in the past year of special interest to the welfare of our Society. In 1925, Messrs. Brunner, Mond & Co. gave a grant of £500 for three years towards the publication fund. In substitution for this grant, Sir Alfred Mond, on behalf of Imperial Chemical Industries, Ltd., has this year given a grant of £1,000 a year, until further notice, to help meet the deficit in the publication funds. This is a very welcome gift to the Society in a direction where help is much needed. Not only has the cost of printing greatly increased since the war, but there has been a notable increase in the number of papers published by the Society. This is specially marked in the 'A Proceedings,' where, in place of one or two volumes per year before the War, three or four volumes now appear, the separate numbers being issued with promptness and regularity.

On the Biological side, where the process of differentiation into separate subjects has probably gone further, the healthy and progressive activities of the specialist societies, which the Royal Society regards with pride as its children, has prevented so large an expansion in our published Proceedings. The expansion on this side of the Society's activities is more clearly shown in the 'Philosophical Transactions,' where the Society is able to do the important service to science of issuing large and elaborately illustrated monographs, the publication of which would be beyond the scope or the resources of the specialist organisations.

Anyone who reads our 'Proceedings' cannot fail to be impressed in general by the great variety and importance of the papers appearing in them. In some respects our Society is now the most important medium of publication of papers in Experimental and Theoretical Physics and Physical Chemistry in this country. This development of the Society's activity owes much to the energy and devoted work of the Physical Secretary, Dr. Jeans. This growth has in many ways thrown a heavy burden on some of the Fellows of our Society, for it is to be remembered that in general each paper has to be reported on favourably by one or two referees before it is accepted for publication. This difficult work of adjudication has been undertaken uncomplainingly by our Fellows, and we are grateful to them for invaluable help in this important matter.

While the growth of the 'A Proceedings' and their punctual publication has led to a marked increase in the sales, there still remains a deficit, which is in part met from the residue of the Government Grant but mainly by special grants in aid from the Messel and Mond Bequest Funds. In no way can our Society be more helpful to the progress of science than by prompt publication of records of important original work, and it would be a great advantage if our Society could have at its disposal ample funds specially allocated for this important purpose.

During the last few years, your officers have given much thought to methods of improving the attendance of Fellows at the ordinary meetings. While special lectures and discussions, and many of the ordinary meetings, are in general well attended, there are occasions when important and interesting papers are read before a very small audience. Quite apart from the painful impression left on the presiding officers, the sparse attendance has inevitably a discouraging effect on the reader of the paper, particularly, as is often the case, if he has come from a distance and spent much time and trouble in order to present the subject matter of his paper in an interesting way. With a view to improvement, the Secretaries in recent years have endeavoured to choose a group of related papers of special interest for reading and discussion at a particular meeting. While this to some extent has been successful in its purpose, no one who has the interests of the Society at heart can feel entirely satisfied with the present state of affairs.

It is only by the co-operation and goodwill of our Fellows that we can hope to remedy this defect in our meetings. If only a small fraction of our Fellows who have a special knowledge of the subject matter of the group of papers to be read at a particular meeting, made a point of attending, we could be sure of an interesting meeting and discussion, profitable alike to all concerned. I would, therefore, urge on our members the duty of attending as far as possible the meetings of our Society, even though it may involve some sacrifice of their time and energy, and even of their inclinations.

In the early days of our Society it was customary for members and their friends to perform experiments of special interest before the Fellows, quite apart from any question of publication of the results. We know from the history of our Society the importance attached to such demonstrations and their value in disseminating information in various branches of science. This custom gradually fell into abeyance, probably as a result of the ever-increasing specialisation of the sciences, and apart from the Soirées, it is only on special occasions in recent years that experimental demonstrations have been made at our meetings.

It has occurred to some of our members that it would be a definite advantage to the Society if the old custom were revived, and the members encouraged to show experiments of special interest and novelty during tea-time, before the beginning of the ordinary meeting. There can be no doubt that such demonstrations would add greatly to the interest of the meetings and might indirectly help to increase the attendance during the reading of papers.

In order to give this suggestion a fair trial, portable demonstration tables have been placed in the tea-room with connections for water, gas, and electric current. In every branch of science, and particularly in the biological sciences, there must be many simple experiments and preparations which can readily be shown in such an extemporised laboratory—demonstrations that would prove of interest not only to the specialist but to the Society in general. I trust that our Fellows will not only avail themselves of these facilities but also encourage their scientific friends to do so.

In the short time at my disposal, I would like to make a few remarks on the results of investigations carried out in recent years to produce intense magnetic fields and high voltages for general scientific purposes. In the past our laboratories have had to be content with the comparatively weak magnetic fields provided by the ordinary electro-magnets and the voltages supplied by simple electrostatic machines and induction coils. In order to push further our investigations in many directions, much stronger magnetic fields and higher voltages are required in the laboratory. Scientific men thus naturally follow with great interest advances in these directions, whether undertaken for purely scientific or for technical uses.

By means of modern electrostatic machines, it is not difficult to produce weak direct currents at potentials from 200,000 to 300,000 volts, while a large well-insulated induction coil can give momentary voltages of a similar magnitude. The wide use of X-rays for diagnostic and therapeutic purposes has led to a marked improvement in apparatus for exciting intense X-rays. The requirement of very penetrating X-rays for deep therapy in our hospitals has led to the construction of comparatively light transformers, which will supply the requisite small currents at voltages between 300,000 and 500,000.

One of the simplest ways of producing very high voltages is by the Tesla transformer, in which the oscillatory discharge of a Leyden Jar is passed through the primary of an air transformer. In this way it is not difficult to produce voltages in the secondary of the order of a million volts, and I understand as much as five million volts have been obtained in the Carnegie Institute at Washington. The striking effects produced by these rapidly oscillating discharges from a Tesla coil, and the immunity with which long sparks may be taken through the body, are well known to all. The rapid frequency of the oscillations and the comparatively small energy given to the secondary of a Tesla coil has, however, restricted its use for general technical purposes as a source of high voltages, although it is now finding an application for the testing of insulating materials.

In order to transmit electrical power economically over long distances, there is a continuous tendency to raise the voltage in the transmission lines. This increase of the operating voltage has led to the need of very high voltages to test the insulating properties of these lines and their transformers and the effect of electric surges in them. In the course of the last few years a number of high-voltage plants have been installed for testing purposes in various countries, which give from one to two million volts. These voltages may be obtained either by a very large well-insulated power transformer or more generally by a cascade method employing several transformers in which the secondary current of one transformer passes through the primary of a second, and so on, the cores of the successive transformers being mounted on insulating pedestals. This cascade method is very advantageous for the purpose, since it allows a great reduction in weight and dimensions of the transformers. Such a high-tension plant in full operation is a striking sight, giving a torrent of sparks several yards in length and resembling a rapid succession of lightning flashes on a small scale. Actually the highest voltage so far obtained by these methods is very small compared with the voltage in a normal lightning flash from a cloud to the earth, where the difference of potential may be as high as a thousand million volts.

There appears to be no obvious limit to the voltages obtainable by the cascade arrangement of transformers, except that of expense and the size of the building required to install them. I am informed that the General Electric Company, of Schenectady, have a working plant giving 2,800,000 volts (max.) and hope soon to have ready a plant to give 6 million volts.

While no doubt the development of such high voltages serves a useful technical purpose, from the purely scientific point of view interest is mainly centred on the application of these high potentials to vacuum tubes in order to obtain a copious supply of high-speed electrons and high-speed atoms. So far we have not yet succeeded in approaching, much less surpassing, the success of the radioactive elements, in providing us with high-speed α-particles and swift electrons. The α-particle from radium C is liberated with an energy of 7·6 million electron volts, *i.e.*, it has the energy acquired by an electron in a vacuum which has fallen through this difference of potential. The swiftest β-rays from radium have an energy of about 3 million electron volts, while a voltage of more than 2 million would be required to produce X-rays of the penetrating power of the γ-rays.

The application of high voltages to vacuum tubes presents serious technical problems, but a vigorous attack on this side of the question has been recently undertaken by Dr. Coolidge, whom we are glad to welcome as one of our Medallists to-day. In 1894, Lenard made the discovery that high-speed cathode rays generated in a discharge tube could be transmitted into the open air through a very thin window, and made many important observations on the laws of absorption of these swift particles. The voltage used to accelerate the electrons in these experiments seldom exceeded 80,000 volts and the rays were stopped in passing through a few inches of air. Taking advantage of the great improvements in vacuum technique and the ease of supply of electrons from a glowing filament, Dr. Coolidge has constructed an electron tube which will stand 300,000 volts, the rays passing into the air through a thin plate of chrome-nickel-iron alloy about 0·0005 inch thick.

It has not so far been found practicable to apply much more than 300,000 volts to a single tube, on account of the danger of a flash over, due possibly to the pulling-out of electrons from the cathode by the intense electric field. For the application of still higher voltages, a number of tubes are arranged in series and communicating with one another, the fall of potential in each being about 300,000 volts. In these preliminary experiments, a large induction coil has been used to generate the voltage. So far experiments have been made with three tubes in series and 900,000 volts, giving a supply of electrons corresponding to one or two milliamperes through the thin window in the last tube. This gives an intense beam of high-velocity electrons, which spreads out into a hemisphere, due to the scattering of the electrons in passing through the metal window and the surrounding air, extending to a distance of about two metres from the window. Marked luminous effects are produced in the air itself and in phosphorescent bodies placed in the path of the rays. I am informed by Dr. Coolidge that further experiments are in progress and it is hoped to extend the system for still higher voltages.

While the energy acquired by the individual electrons in falling through 900,000 volts is smaller than that possessed by the swifter β-particles expelled from radium, the number emitted from the electron tube is very much greater; for example, the number of electrons per second corresponding to a current of 2 milliamperes is equivalent to the number of β-rays emitted per second from about 150,000 grammes of radium in equilibrium.

While important progress has been made in artificially producing streams of swift electrons, there is still much work to be done before we can hope to produce streams of atoms and electrons of a much higher individual energy than the α or β-particle spontaneously liberated from radioactive bodies. As we have seen, the α-particle from radium C is initially expelled with an energy of about 8 million electron volts. So far the α-particle has the greatest individual energy of any particle known to science, and for this reason it has been invaluable in exploring the inner structure of the atom and giving us important data on the magnitude of the deflecting field in the neighbourhood of atomic nuclei and of the dimensions of the nuclei. In case of some of the lighter atoms, the α-particle has sufficient energy to penetrate deeply into the nucleus and to cause its disintegration manifested by the liberation of swift protons.

It would be of great scientific interest if it were possible in laboratory experiments to have a supply of electrons and atoms of matter in general, of which the individual energy of motion is greater even than that of the α-particle. This would open up an extraordinarily interesting field of investigation which could not fail to give us information of great value, not only on the constitution and stability of atomic nuclei but in many other directions.

It has long been my ambition to have available for study a copious supply of atoms and electrons which have an individual energy far transcending that of the α and β-particles from radioactive bodies. I am hopeful that I may yet have my wish fulfilled, but it is obvious that many experimental difficulties will have to be surmounted before this can be realised, even on a laboratory scale.

We shall now consider briefly the present situation with regard to the production of intense magnetic fields. Electro-magnets are ordinarily employed for this purpose and the magnetic fields obtainable are in the main limited by the magnetic saturation of the iron. By the use of large electro-magnets and conical pole pieces, the magnetic induction can be concentrated to some extent. For example, in the large Weiss electro-magnet, a field of 80,000 gauss can be obtained over a volume corresponding to about a pin's head, and a field of about 50,000 gauss through a volume of about 20 cubic mm. In general, however, most experiments have been restricted to fields less than 35,000 gauss.

In order to push this method of obtaining magnetic fields to the practical limit, Prof. Cotton, of Paris, has designed and has under construction a very large electro-magnet. The cross-section of the iron will be of the order of one square metre, and about 500 kilowatts will be required to excite it. Such a large electro-magnet will not give a much stronger maximum field than existing ones, but will produce a field of given intensity through a larger volume. No doubt this electro-magnet will prove very useful in experiments where steady fields of high intensity are required through a reasonable volume.

In order to provide magnetic fields of the order of half a million gauss, the use of the electro-magnet must be abandoned. Some years ago, Dr. Kapitza suggested that intense momentary magnetic fields could be obtained by sending a very strong current through a coil for such a short interval that the heating effect in the coil is restricted to a permissible value. It is well known that momentary currents of great intensity can be produced by the discharge of a large high-voltage condenser through a coil. Experiments of this kind have been made by Dr. Wall, in which the duration of the discharge was of the order of one-thousandth of a second. It is estimated that in this way a field of about 200,000 gauss may be reached.

In his experiments to obtain intense magnetic fields, Dr. Kapitza at first employed a special form of accumulator to send a very strong current through a coil for about one-hundredth of a second, the current if necessary being sharply broken after this interval. In this way it was shown to be practicable to carry out experiments on the Zeeman effect, and in bending α-particles in magnetic fields considerably stronger than those obtainable with ordinary methods. In subsequent experiments, a generator of special design was installed, which gives a very large current, of the order of 70,000 amps. at 2000 volts when short-circuited. A heavy current from the generator is passed for about one-hundredth of a second through a coil and then sharply broken by means of a specially designed automatic break. By this means very strong momentary currents can be produced.

The main difficulty in these experiments has been to construct a coil strong enough to withstand the enormous disrupting forces which arise when a large current is passed through the coil. By special attention to the design, a coil has been constructed which gives a field of 320,000 gauss over a volume of about 3 c.cm. without any signs of fracture. Measurements have been regularly carried out in fields of this magnitude. It is anticipated that the present design of coil will give about 500,000 gauss before bursting, and that still higher fields can be obtained in coils specially constructed for the purpose. An account of these experiments has recently been published in the Society's Proceedings.

As the current only lasts about one-hundredth of a second, oscillograph methods have to be employed to determine the strength of the current and magnetic field. There seems to be no inherent difficulty in conducting magnetic experiments in these momentary fields, for the shortness of the time available is in many cases compensated for by the magnitude of the effects which arise in such intense fields.

The investigations, which have been carried out in the Cavendish Laboratory, Cambridge, have been made possible by the generous support of the Department of Scientific and Industrial Research, which has defrayed the cost of the apparatus and experiments.

The application of these new methods of producing intense fields opens up a wide region of research, where all magnetic properties can be examined in fields 10 to 20 times stronger than those hitherto available. Such researches cannot fail to yield results of great interest and importance and to advance our knowledge of magnetic phenomena.

While the application of external magnetic fields of the order of one-million gauss will no doubt markedly perturb the orbits of electrons in the outer structure of the atom, it is not to be anticipated that it will seriously affect the stability of atomic nuclei. General evidence indicates that the magnetic fields within the nucleus are much too great for such a relatively weak external field to cause a disruption of the nucleus. In this direction, the bombardment by high-speed particles is likely to be far more effective than the strongest magnetic field we can hope to generate.

The advance of science depends to a large extent on the development of new technical methods and their application to scientific problems. The recent work to which I have referred, on the development of methods of producing high voltages and intense magnetic fields, is not only of great interest to scientific men in itself but promises to provide us with more powerful methods of attack on a number of fundamental problems.

The Copley Medal is awarded to Sir Charles Scott Sherrington, O.M., my immediate predecessor in this Presidential Chair.

Sherrington early chose as the special field of his investigations the physiology of the central nervous system. To this, during some thirty years, he has steadily devoted his great skill in experiment, bringing the immense complexities of its function within the range of objective analysis, and revealing fundamental plan and orderly sequence in the reflex actions by which it controls the activities of the body, and continuously adjusts them to the environment. The results of this work have been embodied in a series of some two hundred original memoirs, presenting a continuous record of progressive investigation. The earlier stages have been brought under review and treated synthetically by Sherrington in his now famous Silliman Lectures on "The Integrative Action of the Nervous System." In these he deals with the occurrence and significance of the muscular rigidity which appears when the higher brain is removed, with the co-ordination of muscular movements by reciprocal excitation and inhibition of antagonistic muscles, with the rhythmical, phasic activity which the conflict produces in the centres concerned with certain movements, and with the appearance of a purposeful character which the integrating action impresses on many forms of reflex response.

During more recent years our own Proceedings have borne steady witness to the progress made in the finer analysis of the functions of the central nervous system by Sherrington himself and by pupils using the methods which he has created, and building on the foundations which he has laid.

The influence of Sherrington's investigations has spread far beyond the limits of his own laboratory and has inaugurated a new era in neurological investigation throughout the world. In this connection we may fittingly pay a tribute to the memory of one of the most eminent among those who have drawn inspiration from Sherrington's work and from personal contact with himself. Rudolph Magnus, of Utrecht, whose early death in this year the whole world of science deplores, found there the impulse to his own original and far-reaching investigations, the results of which he embodied in the Croonian Lecture to this Society two years ago, on "Animal Posture." In every civilised country, in the neurological clinic as well as in the laboratory of physiology and of experimental psychology, the influence of Sherrington's work is felt, giving the clue to the understanding of many of the motor symptoms of nervous disorder, and holding out promise that even the higher functions of the central nervous system will not remain permanently beyond the reach of man's experimental enquiry.

A Royal Medal is awarded to Prof. John Cunningham McLennan.

For more than thirty years Dr. J. C. McLennan has been an energetic and enthusiastic experimenter, having published over one hundred papers in the principal scientific publications of England and America. These are mainly concerned with radio-activity, gaseous conduction of electricity, the spectra of the elements, and the liquefaction of gases. In all these subjects he has made substantial and much-used contributions to quantitative knowledge, generally of high accuracy.

Among his works of outstanding merit may be mentioned the measurements he has made with his pupils on the fine structure of spectral lines, which are of much importance to modern theories of the mechanism of the atom. Recently he has had a sensational success in tracing to its source the elusive auroral line λ 5577, an extremely difficult task which had baffled the skill of many previous investigators. This is important not only in itself but on account of the information it yields as to the structure of the upper atmosphere.

Apart from his own researches, he has built up a most efficient School of Physics in Toronto, and is largely responsible for the present strong position of physical science in Canada. He has devoted much energy to the establishment of a cryogenic laboratory in Toronto, a heavy task which he has carried out with much success.

During the war, he was in charge of important scientific work for the Admiralty; he developed with great energy the supply of helium from the natural sources in Canada and the States, and rendered material assistance in connection with anti-submarine warfare, especially in mining operations on a large scale and of novel character.

A Royal Medal is awarded to Sir Thomas Lewis.

From 1911 onwards to the present day, Sir Thomas Lewis has taken a leading part in the remarkable growth of our knowledge of the mammalian heart-beat, which has been one of the conspicuous scientific achievements of the period in question. Until he began his work, nothing was known for certain of the relations of the specialised structures known to anatomists with the origin and propagation of the beat of the heart. Lewis's researches enabled him to locate the point of origin of the beat, and to plot out the course of the wave of excitation over the ventricles and auricles of mammals. By extending these observations to the hearts of representative vertebrates, he was able to compare the modes of spread of the wave with the special forms of the electrocardiagram, and thus to appreciate clearly the meanings of the several deflections. Further extension to diseased hearts led to the interpretation of the abnormalities of the electrocardiagraphic record.

In 1911 Lewis was able to show that, as Cushny had previously suggested, certain cardiac irregularities are due to fibrillation of the auricles; and his later clinical and experimental work on auricular fibrillation and flutter suggest that the irregularities are due to the formation of an endless circulating wave of contraction in the auricles.

In this special field of physiology and pathology, of such great importance to medicine, Lewis's researches have replaced a mass of scattered, suggestive observations by a coherent and established theory. His book on this subject, which is necessarily based, to a large extent, upon his own observations, is recognised as authoritative throughout the world.

Quite recently Lewis has published another book which embodies the results of investigations of the peripheral circulation, upon which he has been engaged during the past twelve years. The response to other kinds of stimulus closely resembles that invoked by injection of histamine into a puncture, and Lewis produces strong evidence that even the response to pressure is due to the liberation in the tissue of a chemical compound, which, if not actually histamine, resembles that substance very closely in its physiological effects.

In these investigations, which may be taken as models for the application of exact methods to human physiology and pathology, Lewis shows the same qualities of accurate experimentation and exact reasoning which are so conspicuous in his work upon the heart's action.

The Davy Medal is awarded to Prof. Arthur Amos Noyes.

Prof. Noyes was the torch-bearer of the modern theories of solution to the West, and under his guidance there grew up in the Massachusetts Institute of Technology a school of research in physical chemistry which held the leading place in America. His researches have been chiefly concerned with the properties of solutions, in particular of electrolytic solutions. Soon after the inception of the electrolytic dissociation theory of Arrhenius, it was recognised that all was not well with the strong electrolytes. Whilst qualitatively their properties were accounted for by the theory, there yet existed marked quantitative discrepancies. Accurate measurement of the properties of such solutions was the first requisite for the attack on the problem, and to this task Noyes applied himself. His investigation of the conductance of aqueous solutions up to temperatures as high as 300° forms a classical example of exact physico-chemical measurement executed under conditions of great experimental difficulty.

His work on the influence exerted by one salt on the solubility of another, on transport numbers and the mobilities of the ions, on the ionisation of pure water at different temperatures, is all directed to the same end. Noyes showed the importance of the classification of the strong electrolytes according to their valency type, and over twenty years ago attempted to take into account the electrostatic forces between the ions. He thus foreshadowed the modern theory now so widely developed by Noyes himself amongst other workers.

Outside this field Noyes made many additions to our knowledge of solutions; for example, the mass-action theory of acid indicators, velocities of reactions of different orders, and reaction-velocity in heterogeneous systems. Noyes has exercised a great influence on physical chemistry, not only by the value of his experimental work, but by his careful analysis of the fundamental concepts of the science, and by his clear and logical presentation of their nature and their inter-relations.

The Buchanan Medal is awarded to Dr. Major Greenwood.

Dr. Greenwood, Professor of Epidemiology and Vital Statistics, University of London (London School of Hygiene), is specially distinguished for the statistical study of medical subjects, having applied the statistical method to the elucidation of many problems of physiology, pathology, hygiene and epidemiology. He has been pre-eminent in encouraging and developing the use of modern statistical methods by medical laboratory investigators and in securing the adequate planning and execution of field investigations. He is almost unique in the possession of both the medical knowledge and mathematical ability which are essential in these researches. Dr. Greenwood is the author (with Prof. E. L. Collis) of a book on 'The Health of the Industrial Worker,' and of numerous biometric studies dealing with the causation, prevention and treatment of disease.

The Hughes Medal is awarded to William David Coolidge, a distinguished member of the scientific staff of the General Electric Company of America.

Science is under a great debt to Dr. Coolidge for the invention and production of a new type of X-ray tube, called by his name, of great flexibility and power, which has proved of great service not only in Medical Radiology but in numerous scientific researches. In the last few years he has applied his unrivalled technical knowledge to the generation of high-velocity cathode rays, which can be passed into the air through a thin window as in Lenard's pioneer experiments thirty years ago. Such researches are of great importance to science as they promise to provide us with new methods of obtaining a copious supply of swift electrons and high-speed atoms of matter for experimental investigations.

The Action of Guanidins on the Melanophores of the Skin of the Frog (Rana temporaria).

By S. Ochoa.

Communicated by Prof. D. Noël Paton, F.R.S.—Received October 2, 1927.)

(From the Physiological Laboratory, University of Glasgow.)

[Plates 19 and 20.]

The part played by the methylguanidins in metabolism has been recently considered by Noël Paton (1), who pointed out, *inter alia,* their effect on the tone of skeletal muscle, and adduced evidence to show that the increased tonicity in tetania parathyreopriva is associated with an increase of these substances in the blood (1A). Within the past year this has been confirmed by Kühnau (2). The work by Frank, Stern and Nöthmann (3) indicates that these methylguanidins have an activating effect on the sympathetic nerve-endings, a conclusion supported by the effect on the pupil described by Putseys and Swaen (4).

A full consideration of the physiological action of the guanidins is given by Führer in Heffter's 'Handbuch der exper. Pharmak.' (vol. 1, p. 684), Berlin, 1923, with a bibliography; detailed references to the older work are therefore unnecessary.

In the course of an investigation on the influence of the administration of guanidins on the creatin content of muscle in frogs, it was observed that the colour of the animal became distinctly paler. An action upon the melanophores of the skin was indicated, and this has been investigated. The injections were made for me by Drs. Campbell and Watson, to whom my thanks are due.

1. *Action of Guanidins on the Intact Frog.*

The frogs were placed in glass containers, to which some water was added to provide moisture, and the containers themselves placed in the shade against a dark background. In these conditions, according to Hogben (5), the tendency of the melanophores is to remain expanded, so that the action of a melanophore-contracting substance may be studied under favourable conditions. The mouth of the containers was only partially closed to allow free ingress of air. No special care was taken as regards temperature, which varied little in the laboratory, since moisture and shade are sufficient to keep the frogs dark. In all cases control frogs were placed in the same conditions as those subjected to experiment.

As to normal melanophore reactions, my own experience is that dryness very soon induces pallor, although the frog replaced in wet conditions soon recovers its dark appearance if it has not been kept in dryness for long and the lightening induced is not very pronounced. Light and darkness seem to act more slowly in promoting their respective reactions, as is pointed out by Hogben (5).

Frogs (*R. temporaria*) weighing about 20 to 25 gms. were used, and the experiments were carried out in July and August. In the first experiments every frog received 10 mgm. of methyl-guanidin hydrochloride* into the dorsal sac. Later it was observed that the lightening of the skin was equally well induced by smaller doses. 1 mgm., injected either into the dorsal sac under the skin, or intraperitoneally, is almost as powerful in promoting lightening as are 10 mgm., though the effects are not so rapidly marked. In one case a fairly pronounced pallor, as compared with a normal dark frog, was induced by the injection of 0·5 mgm. With 1 mgm. the lightening begins to be observed within from 7 to 15 minutes after the injection, reaching its maximum after about 45 minutes to one hour, when the pallor induced by the drug contrasts strikingly with the dark appearance of the control frog (Plate 19, figs. 1, 2 and 3).

More than 30 frogs have been injected up to date, and in all cases the reaction could be observed. The duration of the pallor brought about by the guanidin has not been accurately determined; it averages 4 hours when 1 mgm. has been injected, but it must be pointed out that individual variations are observed within rather wide limits. The web of the foot under the microscope showed the melanophores to be in a contracted state as compared with the expanded, reticulated condition in the control frog (figs. 4 and 5).

The same effects were observed by injecting guanidin hydrochloride or dimethyl-guanidin hydrochloride. The frogs also always developed quite marked muscular symptoms along with the characteristic lightening of the skin. In the case of dimethyl-guanidin the onset of the general symptoms and lightening seem to be slower, though their duration is greater. Further experiments will be carried out to elucidate this point.

The work of Hogben and others suggested the possibility that this action might be due to a stimulation of the suprarenals and an increase of adrenalin in the blood, which is known to contract the melanophores. That this is not the explanation is shown by the fact that the contraction occurs in the amputated leg.

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* The salts of methyl- and dimethyl-guanidin were supplied by 'British Drug Houses.'

2. *Injection into the Amputated Leg.*

A dark frog was killed, and the hind legs amputated and placed in Ringer's solution in small beakers. 10 mgm. of either guanidin, methyl- or dimethyl-guanidin hydrochloride were injected into the gastrocnemius of one of them, the other remaining as a control. The webs were examined under the microscope at various periods. Sometimes the change in colour was observed with the naked eye.

Generally after half an hour, and sometimes before, the melanophores of the control leg began to contract (*post-mortem* contraction), but the action of the guanidins could be detected before the natural contraction occurred. As an example, I quote one experiment out of five carried out following the same general scheme:—

Experiment 4.

Hind limbs amputated from a normal dark frog.
- (*a*) Injection of 1 c.c. 1 per cent. guanidin (10 mgm.).
- (*b*) Control.

Placed in Ringer's solution.

10 minutes after (*a*) is much lighter than (*b*).
- (*a*) presents pronounced spontaneous twitchings.

Webs microscopically observed after 15 minutes.
- (*a*) Melanophores contracted.
- (*b*) Melanophores expanded, displaying a reticulate condition.

After 30 minutes limb (*b*) is becoming lighter, but microscopical examination of the webs shows that there is still a difference between the melanophores from (*a*) and (*b*), those from the latter being expanded (Plate 20, figs. 6 and 7).

3. *The Isolated Web of the Foot.*

The web of the foor was cut with scissors from the toes, and placed in Ringer's solution or the guanidin solutions to be tried, being examined microscopically at various periods afterwards. As in the previous experiments, guanidin, methyl- or dimethyl-guanidin were used, either in Ringer's or in aqueous solution. In some experiments either 0·1 c.c. or a drop of pituitrin was added to the bottles containing 1, 2, 4 or 5 c.c. of fluid.

The following experiments may be quoted out of seven carried out:—

Experiment 1.—Normal dark frog.

Webs of the foot placed in two bottles (*a*) and (*b*), containing:—
- (*a*) 0·5 c.c. of 1 per cent. methyl-guanidin.
- (*b*) Ringer's solution (control).

30 minutes later microscopic observation:—
- (*a*) Melanophores contracted.
- (*b*) Melanophores expanded (reticulate).

Experiment 4.—Normal dark frog.

Webs placed in three bottles—(*a*), (*b*) and (*c*)—containing:—

(*a*) 1 c.c. Ringer's solution plus 0·1 c.c. pituitrin (Parke Davis).

(*b*) 1 c.c. 1 per cent. guanidin plus 0·1 c.c. pituitrin.

(*c*) 1 c.c. 1 per cent. methyl-guanidin plus 0·1 c.c. pituitrin.

1 hour later microscopic examination:

(*a*) Melanophores fully expanded.

(*b*) Most of them expanded, but many contracted.

(*c*) Same as (*b*).

The difference between (*a*), (*b*) and (*c*) is fairly well marked. No further change could be observed 24 hours afterwards.

Thus, even in the case of presence of pituitrin, the guanidins exert their melanophore-contracting action, though in this case the action is much weakened.

Melanophores of normal webs placed in Ringer's solution contract normally in less than an hour. The action is thus a direct one, either directly on the melanophores or through the nerve terminations in them (Plate 20, fig. 8).

Hogben states that there is no definite histological evidence that the melanophores of the frog are connected with the nervous system, and he records a number of experiments to prove that they are not influenced by nerves.

I am informed by Prof. Noël Paton that Mr. Edward S. Russell, M.A., D.Sc., working in this laboratory in 1909, was able to demonstrate by staining with methylene blue the termination of nerve-fibres in the melanophores of the mesentery of the frog. In reply to a letter Mr. Russell writes: "I did not publish anything on the innervation of the melanophores in the frog. I wish now I had, as my results were fairly definite. . . . Some preparations certainly showed fine nerve-fibres apparently entering the melanophores."

The early work of Lord Lister afforded clear evidence that the state of the melanophores is modified through the nervous system. It is therefore probable that the action of guanidins on these melanophores is through the endings of the sympathetic nerves, as it is in other situations in the body. The action of adrenalin favours this view.

4. *The Antagonism of Calcium Salts.*

The action of calcium salts in counteracting the symptoms of tetania parathyreopriva is well known. That they also antagonise the effects of the guanidins was first shown by Führer (6), and has been amply confirmed by subsequent investigations (Noël Paton and Findlay (*op. cit.*, p. 336), Burns and Watson (7), Major and Stephenson (8), Kühnau and Nöthmann (9)). It seemed, therefore, of interest to study the influence of calcium salts upon the action of the guanidins on the melanophores.

It has been found that if a solution of calcium lactate or chloride is injected into the dorsal sac of a frog previous to the injection of guanidin, the frog does not change in colour under the influence of the latter, or becomes but very slightly lighter, contrasting strikingly with a control frog injected with guanidin only (Plate 20, fig. 8). Very often a strong lightening of colour is induced by the guanidin, in a circumscribed spot where the injection of the guanidin has been made, when the frogs were previously injected with calcium. This is no doubt a proof that the calcium has actually checked the effect of the guanidin.

On the other hand, if the calcium injection is made on a frog during the pallor induced by the guanidin, it recovers its normal dark hue much sooner than a control frog to which no calcium has been injected. In all cases the calcium either inhibits the appearance of tremors, or makes them disappear when they are already established by a previous injection of guanidin.

Generally the calcium salts were injected in doses from 2 to 5 mgm., and the guanidin (guanidin, methyl- or dimethyl-guanidin) in doses from 1 to 2 mgm. Sometimes a repetition of the calcium injection had to be made when the frog was observed to become lighter in spite of a previous injection.

Fourteen experiments have been carried out with uniform results. One of them may be quoted here :—

Experiment 5.—August 16, 1927.

Four normal dark frogs, (*a*), (*b*), (*c*) and (*d*).

11.30 a.m.—The frogs are injected as follows :—

(*a*) 0·4 c.c. Ringer's solution, 5 minutes later 1 mgm. methyl-guanidin.
(*b*) 0·4 c.c. 1 per cent. $CaCl_2$ (4 mgm.), *5 minutes later* 1 mgm. methyl-guanidin.
(*c*) 1 mgm. methyl-guanidin.
(*d*) 1 mgm. methyl-guanidin.

(Injections into the dorsal sac.)

(The frogs are placed in glass containers with some water, and covered with black paper.)

11.45 a.m. Frogs (*a*), (*c*), (*d*), tremors. Frog (*b*) no tremors.

12 noon. Frogs (*a*), (*c*), (*d*), pale. Frog (*b*) dark. Only a light spot is shown at the point where the injection was made. (Plate 20, fig. 8.)

The difference is quite well marked between frog (*b*) and frogs (*a*), (*c*), (*d*).

12.13 p.m. Frog (*c*). Injected 0·4 c.c. 1 per cent. $CaCl_2$ (4 mgm.).
Frog (*d*). Injected 0·4 c.c. Ringer's solution.

12.20 p.m. Frog (*c*). No tremors. These are very marked in frogs (*a*) and (*d*).
Frog (*b*). Dark, normal looking.

12.55 p.m. Frog (*c*) is becoming darker. Darker than (*d*), which is still very pale

Experiment 5—(continued).

1.30 p.m.	Frog (*a*).	Recovered dark colour. Tremors still present.
	Frog (*b*).	Normal, dark throughout the experiment.
	Frog (*c*).	Recovered dark colour. No tremors.
	Frog (*d*).	Quite pale. Marked tremors.
2.50 p.m.	Frog (*c*).	Dark, normal looking.
	Frog (*d*).	Still pale.

Calcium itself does not act upon the melanophores—*i.e.*, it does not induce any expansion of these structures. Several experiments have been made on frogs kept pale by placing them in dry containers, and in an illuminated and warm place. Neither calcium chloride nor lactate were observed to promote any darkening of such frogs, injected in doses varying from 4 to 10 mgm., as compared with other non-injected control frogs.

A few experiments have been made to see whether the parathyroid hormone, prepared according to Collip's method, has any influence upon the action of guanidins in frogs. If this hormone raises the blood calcium level, it was to be expected that it would antagonise the effects of the guanidin, in those cases, at least, in which the calcium salts counteract the action of the guanidins.

It is rather surprising that Collip himself (10) failed to find any antagonism between his hormone and guanidin. Major and Buikstra (11) found that the hormone lowers the blood pressure previously raised by guanidin, and recently Nöthmann and Kühnau (12) found that it counteracts the symptoms produced by dimethyl-guanidin.

No definite conclusion can yet be drawn from my experiments, which were interrupted by my departure for Madrid, but it seems that the hormone relieves the toxic action of the guanidins on the melanophores of the frog, and that the lightening of colour induced by these substances in frogs' skin is less pronounced and lasts a shorter time in frogs treated with the parathyroid preparation than in control frogs. Calcium seems to act more rapidly and powerfully in checking the action of the guanidins. This would support the belief that the parathyroid hormone may act in this case by raising the calcium content of the blood.

More experiments will be carried out to elucidate this point further.

Summary.

1. The guanidin hydrochlorides—guanidin, methyl- and dimethyl-guanidin—cause contraction of the skin melanophores in the frog.

2. Evidence has been adduced to show that this action is a direct one, either on the melanophores themselves or through nerve endings in them.

3. Calcium salts antagonise this effect of guanidins just as they do many of the other effects.

4. It is probable that the parathyroid hormone of Collip also antagonises the action of guanidins on the melanophores, perhaps through its calcium-raising power. Further experiments are needed to elucidate this point.

It is my desire to acknowledge my indebtedness to Prof. Noël Paton for allowing me to work in his laboratory, and for his inestimable help and guidance in carrying out the work.

All the photographs and microphotographs have been taken for me by Mr. John Bell, to whom my best thanks are due.

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(11) Major and Buikstra, 'Johns Hopkins Hosp. Bull.,' vol. 37, p. 392 (1925).
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DESCRIPTION OF PLATES.

PLATE 19.

FIG. 1.—Normal dark frogs.

FIG. 2.—Same frogs 35 minutes after injecting that on the right with 1 mgm. methylguanidin into the dorsal sac.

FIG. 3.—Same frogs 23 hours later.

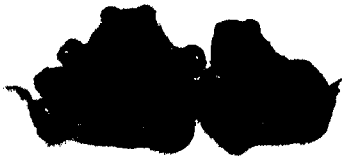
FIG. 4.—Melanophores of normal frog (× 75).

FIG. 5.—Melanophores of frog 40 minutes after injection of 5 mgm. methylguanidin.

PLATE 20.

FIG. 6.—From hind leg of a frog left in Ringer's solution for 30 minutes after amputation. (× 75.)

FIG. 7.—From the other leg, same conditions, but 10 mgm. methylguanidin injected into gastrocnemius. (30 minutes later.) (× 75.)



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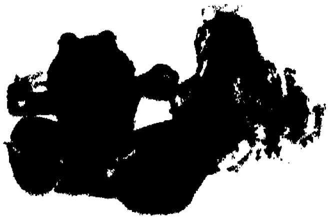

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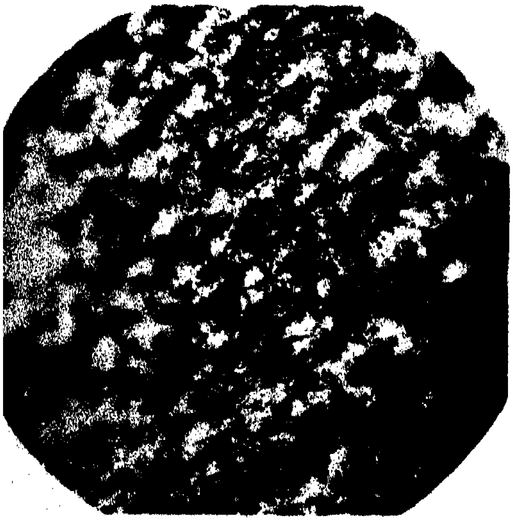

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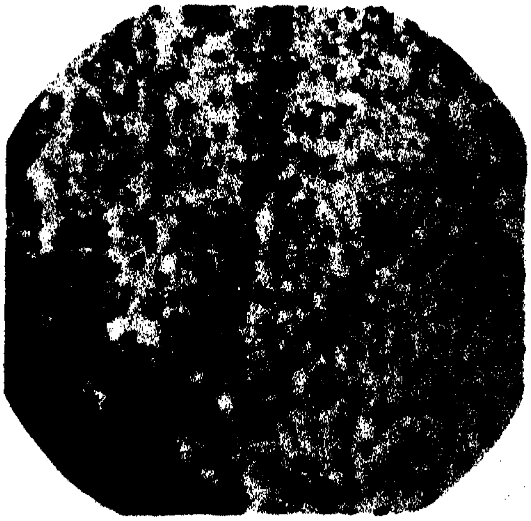

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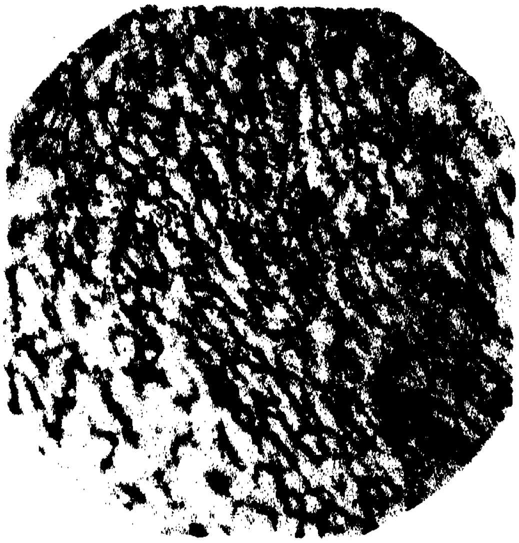

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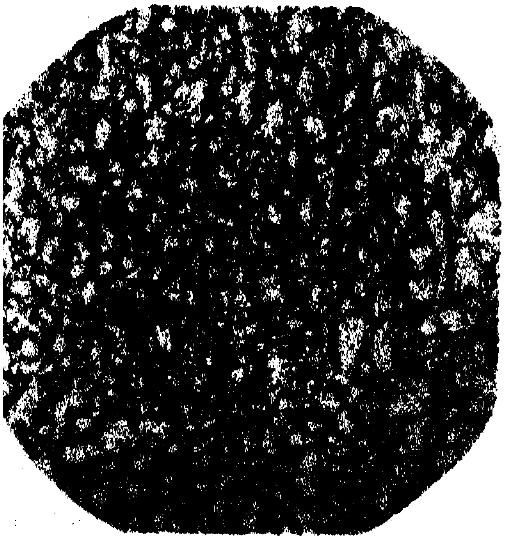

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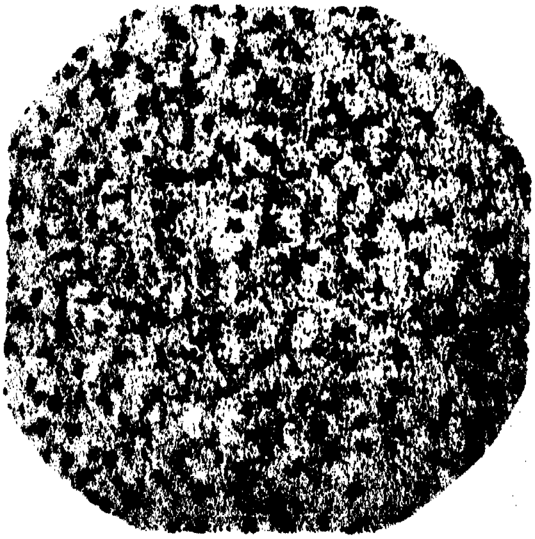

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FIG. 8.—Frog on left injected with 0·4 c.c. 1 per cent. calcium lactate—10 minutes later 1 mgm. methylguanidin. Frog on right injected 0·4 c.c. Ringer's solution. 10 minutes later, 1 mgm. methylguanidin. (Injections in dorsal sac.) Photographed 50 minutes after injection of the methylguanidin.

FIG. 9.—From frog injected with 0·5 c.c. 1 per cent. $CaCl_2$ 5 minutes after, 0·5 c.c. 0·1 per cent. guanidin (1 mgm.). (× 75.) Killed one hour later.

FIG. 10.—From frog injected with 0·5 c.c. Ringer's solution. 5 minutes after, 0·5 c.c. 0·2 per cent. guanidin (1 mgm.). (× 75.) Killed one hour later.

The Formation of Indigotin from Indol by Soil Bacteria.

By P. H. H. GRAY, M.A., Rothamsted Experimental Station.

(Communicated by Sir John Russell.—Received October 5, 1927.)

[PLATE 21.]

Indol ($C_6 H_4 CH : CH . NH$) has a wide distribution in nature, being present in several plants and also produced by putrefactive changes in the intestine. There is no evidence as to its fate after decomposition in soil or in sewage. Its non-persistence at the moderately high temperatures of the manure heap may be due to its volatility; its disappearance from sewage and manured soil may have to be assigned to some other cause.

Supniewski* states that *Bacillus pyocyaneus* can decompose indol with the production of anthranilic (*o*-aminobenzoic) acid, although the action is extremely slow. In attempting to isolate from soil by the usual selective method of cultivation bacteria capable of destroying indol, it was found that in mineral salt solution containing indol, the latter rapidly disappeared, some colourless compounds being formed whose nature has not been determined. Two organisms have, however, been isolated that can decompose indol with the formation of blue crystals. The chief cultural characters of these two organisms are described below.

1. *Pseudomonas indoloxidans* n. sp. *Morphology* in nutrient broth: straight cells 1 μ to 9 μ long, majority about 3 μ long by 1 μ broad. Motile, with 1 to 4 short curved polar flagella (see fig. 1). The vegetative cells are Gram negative but take the usual dyes. Endospores are not formed. The organism is aerobic and grows best at 25° C. to 28° C.

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* 'Biochem. Zeitschr.,' vol. 146, p. 522 (1924).

Nutrient agar colonies, (peptone 0·5, lemco 0·3, agar 1·5 per cent.). *Surface colonies*, (2 days at 28° C.) 1 to 2 mm., round ; convex ; white ; watery ; transparent border, structure radiate, edge erose. (5 *days*) 4 to 5 mm. irregularly round ; slightly convex ; white ; slightly ringed and radiate, shining ; wide translucent border ringed, radiate ; edge undulate. *Deep colonies*, (2 *days*) lens-shaped, brown, slightly irregular. (5 *days*) oval, brown, edge slightly cleft by radiate structure, or lens-shaped.

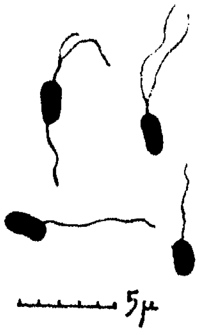

FIG. 1.—*Pseudomonas indoloxidans*, 24 hours old culture on nutrient agar.

Nutrient agar slope (3 *days*) filiform ; convex whitish ; smooth shining ; clear border, undulate.

Nutrient gelatine colonies (peptone 1·0, lemco 0·5, NaCl 0·5, gelatine 12 per cent.). *Surface colonies*, (8 *days* at 14° C. to 19° C.) 1 mm., round ; convex ; light buff ; smooth, shining ; edge erose. Under ⅓-inch objective, structure appears grumose, deeper part rosettiform. *Deep colonies*, rosettes, erose edge.

Nutrient gelatine stab. Nail head.

Nutrient and *peptone broth*, growth cloudy ; indol is not produced.

Carbohydrate broths, (peptone 1·0 per cent., and 1·0 per cent. of the carbon compounds, dextrose, sucrose, lactose, maltose and glycerol). Growth cloudy but no acid or gas produced ; the media become more alkaline than uninoculated controls.

Starch, (nutrient agar + 0·2 per cent. soluble starch). (16 *days*) no diastase produced.

Nitrates, (nutrient broth + 0·1 per cent. KNO_3). (3 *days*) reduced to nitrites. No gas produced and no growth in the closed arm of the fermentation tubes by the sixteenth day.

Aromatic compounds, phenol and *m*-cresol are not attacked.

Indol, decomposed with formation of blue crystals in mineral salt solution and agar medium.

Indol agar, *surface colonies*, (3 *days*) 0·9 mm. to 1·0 mm., flat, whitish intermixed with blue, with central opaque nucleus. Blue particles are found up to about 3·75 mm. from edge of colony. (10 *days*) 2 to 3 mm. diameter ; round ; convex ; white, blue tinged ; smooth, shining ; edge erose ; colony mixed with and surrounded by opaque hour-glass shaped particles. *Deep colonies*, (10 *days*) lens-shaped, brown with blue core and surrounded with blue particles. (22 *days*) in thicker gels the edge of the colony is produced to form a flattened whitish ring.

Indol agar slope. First traces of blue are seen within 20 hours, before any growth is visible.

Indol agar stab. After 48 hours the blue zone extends down to about 5 mm. from the surface.

Isolation. One strain from a soil sample obtained from the Italian Tyrol. (Thanks are due to Miss M. D. Glynne, M.Sc., for having collected this sample.)

2. *Mycobacterium globerulum* n. sp. *Morphology* in nutrient agar: curved cells 2 μ to 9 μ long, majority about 5 μ long and 1 μ broad (see fig. 2) breaking up into chains of coccoid cells about 1 μ diameter (see fig. 3). Non motile.

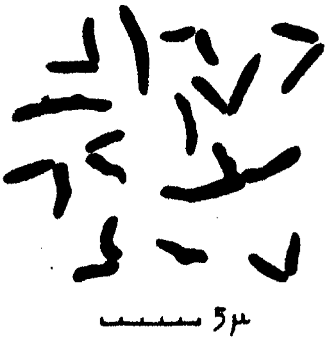

Fig. 2.—*Mycobacterium globerulum*, 20 hours old culture on nutrient agar.

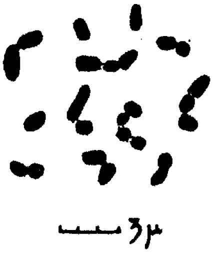

Fig. 3.—*Mycobacterium globerulum*, after 3 days growth on nutrient agar.

Cells are Gram positive but not acid-fast. Capsules can be demonstrated by Muir's capsule method (see Plate 21, fig. 1). On indol agar some cells are surrounded by blue pigment, giving the appearance of capsules (see Plate 21, fig. 2). Endospores are not formed. The organism is aerobic and grows best at 25° C. to 28° C.

Nutrient agar. Surface colonies, (4 *days*) 3 to 5 mm., irregularly round; convex; white; smooth, shining; edge undulate, erose. (7 *days*) more convex and of a watery appearance. *Deep colonies,* (4 *days*) lens-shaped.

Nutrient agar slope, (3 *days*) filiform; flat; watery; edge irregular.

Nutrient gelatine, (19 *days*). *Surface colonies,* 1 to 2 mm., irregularly round; convex; light buff; smooth, shining; edge entire. *Deep colonies,* round, with entire edge.

Nutrient gelatine stab, (8 *days*) nail head, irregularly round; convex; pinkish white; smooth, shining; line of stab erose.

Nutrient and peptone broth, growth cloudy, with viscous suspension. Indol is not produced.

Carbohydrate broths, growth is cloudy in dextrose, sucrose and glycerol, clear with scum or granular suspension in lactose and maltose. No acid or gas produced from any of the compounds.

Starch, (16 *days*) diastase is produced.

Nitrates, (3 *days*) no nitrite or gas produced. No growth occurs in closed arm of fermentation tube.

Aromatic compounds, phenol is attacked, but *m*-cresol is not attacked.

Indol, blue particles are produced on indol agar but not in solution.

Indol agar, surface colonies, (4 *to* 7 *days*) 1 to 2 mm., round ; convex ; white, tinged with blue ; smooth, shining ; entire edge. The blue tinge is chiefly due to discrete hour-glass shaped particles. *Deep colonies*, lens-shaped with bunchy filamentous lateral projections.

Indol agar slope, filiform ; white (later tinged with blue) or buff to yellow ; smooth, shining.

Isolation. From a soil obtained from Cheshire, England, after treatment with scatol.

Bacteria that produce blue pigments on various organic media are known to occur ; the nature and source of the pigments have not, however, in any case been made known. In order to ascertain whether the property of producing a blue pigment on synthetic media containing indol was possessed by other known bacteria, slope cultures of 74 strains of soil bacteria were made on a medium consisting of mineral salt agar with glycerol 0·1 per cent., $(NH_4)_2SO_4$ 0·05 per cent., and indol 0·005 per cent. Very few of these made any appreciable growth in 7 days at 27° C. Only *Ps. indoloxidans* and a new species of *Micrococcus** formed blue particles, but this latter species has not so far formed them in solution cultures. It was decided, therefore, further to investigate the nature of the blue substance produced in cultures of *Ps. indoloxidans*.

The Nature of the Pigment.

The blue compound that appeared in cultures containing *Ps. indoloxidans* was found as insoluble particles ; their identity with indigotin was suggested by the following tests† :—

1. Insolubility in water, alcohol, ether, xylol, benzol.
2. Dissolved in strong sulphuric acid to give a blue solution.
3. This solution, on dilution, dyed silk blue.

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* A description of this organism (*Micrococcus piltonensis*) is in course of publication in 'Centralblatt für Bakteriologie,' II. Abt., Bd. 73.

† I am indebted to Dr. F. Tattersfield for assistance in these tests ; the spectrophotometry was carried out by Dr. S. Judd Lewis, Staple Inn Buildings, London.

4. The colour disappeared on treatment with reducing agents and caustic soda, but returned on pouring into water and on exposure to air.
5. The colour was discharged by nitric acid.
6. A solution in concentrated H_2SO_4 was treated with strong soda, whereupon a green colour appeared ; the colour went to yellow on addition of more soda ; this yellow then went to green on the addition of ammonia.
7. Spectrophotometric analysis of a solution prepared from some of the dry material with concentrated H_2SO_4 produced a curve for the extinction coefficient that was so similar to that obtained from a solution of indigotin di-sulphonic acid prepared from pure powdered indigotin that the identification of the blue substance as indigotin is placed beyond doubt (see fig. 4).

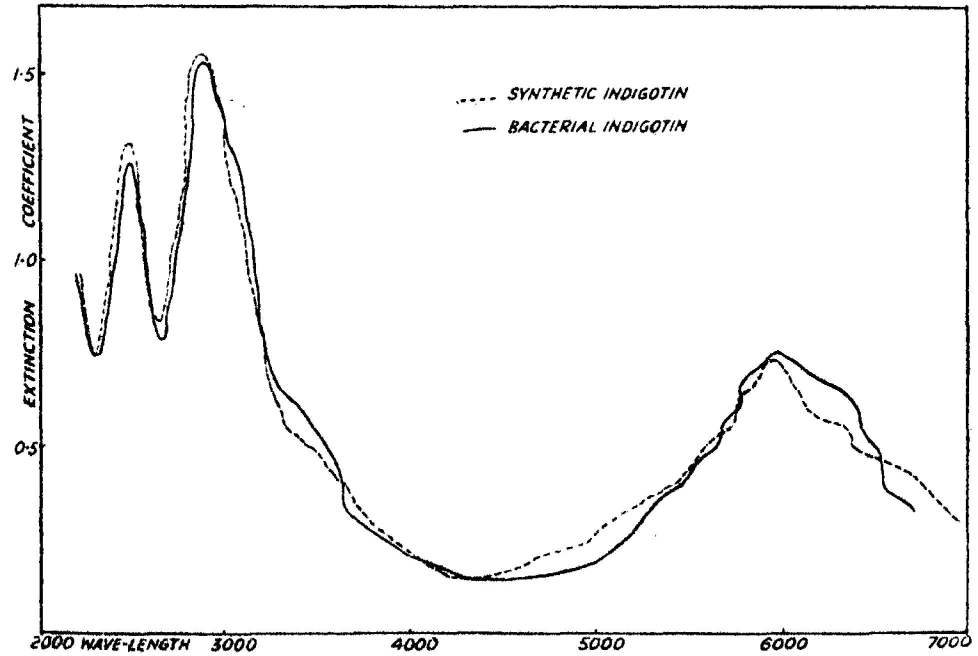

FIG. 4.—Comparative curves for extinction coefficient of synthetic indigotin and indigotin produced from indol by *Ps. indoloxidans*.

The Mode of Formation of Indigotin on an Agar Medium.

Pure indol, melting point 52° C., obtained from the British Drug Houses, was added to a mineral salt agar, after the latter had melted, until the indol was completely dissolved. The following were the salts used in the medium :—

	Per cent.		Per cent.
K_2HPO_4	0·100	$CaCl_2$	0·010
$MgSO_4 . 7H_2O$	0·020	$FeCl_3$	0·002
NaCl	0·010		

To this medium carbon and nitrogen sources were added as glycerol 0·1 per cent. and $(NH_4)_2SO_4$ 0·05 per cent. respectively. The concentration of indol was 0·01 per cent. The medium was then filtered through cotton-wool, tubed, and sterilised in the autoclave at 15 lbs. pressure for 15 minutes.

The general appearance of the growth on indol agar has already been described (p. 264). The particles are first visible as thin needle-shaped blue crystals, about 1 μ long and 0·2 μ wide, with a constriction in the middle and generally fanning out at the ends to about 0·75 μ wide. This structure appears to be the basis upon which the full-sized particle is built. After 20 hours' growth on agar, the particles measure not more than 3 μ long and about 2 μ wide, the appearance being that of a dark blue hour-glass, with a lighter constriction (see Plate 21, fig. 3). On agar media they appear to reach a maximum size of 6 μ long by 3 μ wide, the constriction sometimes disappearing under the heavier deposition of the dye. It is this type of particle with which the curves in figs. 5, 6 and 7 are concerned. Lighter blue flakes, oval in shape and measuring about 10 μ long by 5 μ wide, are produced in liquid media and on the surface of the water at the base of test-tube cultures; often larger irregular crystalline particles appear on the surface of agar platings (see Plate 21, fig. 4). In liquid cultures the accretion of the blue continues until single particles, fan- or fern-shaped, measure about 150 μ long.

Two modes of action whereby the indigotin is formed appear to be possible :—

1. By the production of an exo-enzyme which diffuses out from the growth, producing the reaction in the surrounding medium.
2. By the formation within the cells of a soluble product intermediate between indol and indigotin which diffuses outward and is then converted into indigotin.

Indoxyl, a product intermediate between indol and indigotin, is an unstable compound and is very rapidly oxidised to indigotin on exposure to atmospheric oxygen. On the second hypothesis, if indol were taken in by the organism and excreted as indoxyl, it would be expected that each bacterial cell would become surrounded by a blue " capsule " of indigotin, in the way that has been described

(p. 266) as happening sometimes to cells on indol agar slope cultures of *Mycobacterium globerulum*. This does not take place with *Ps. indoloxidans*, nor does indigotin occur inside the cells under what appear to be the optimum conditions for oxidation. It seems unlikely, therefore, that indoxyl is formed within the cells of this organism.

Production of Indigotin in Agar Stab Cultures.

The production of indigotin in stab cultures is confined chiefly to the top 3 to 5 mm., and extends radially from the line of stab to the walls of the tube. No increase in intensity of blue or depth to which the blue extended has been observed to take place after about the third day. Although the organism continues to grow to some extent on the surface of the stab, it is found that a considerable growth of the organism also occurs along the line of stab. By cutting transverse sections across a stab culture, it was found possible to count the number of crystals of indigotin produced by the organism at different depths of agar. In fig. 5 are shown the results of counts made from the line of stab outwards at two depths, namely, 4 to 5 mm. and 6 to 7 mm., from the surface of a stab culture after 2 days' growth at 25° C.*

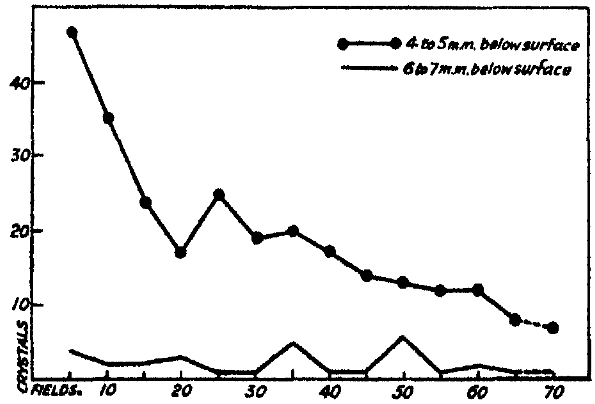

Fig. 5.—Curves showing the number of particles of indigotin produced by *Ps. indoloxidans* at two depths of a stab culture on indol agar.

* The particles were counted as follows. The agar was removed from the test-tube by breaking the latter and a section about 2 mm. thick cut at the fourth to fifth millimetre from the surface. This section was applied to a coverslip and the latter inverted on the mechanical stage of the microscope. The $\frac{1}{6}$-inch objective was focussed on to the edge of the bacterial growth; a cover slip upon which had been marked a rectangle measuring 3 mm. square was placed in the ocular. The number of particles of indigotin within the rectangular area at 50 μ from the surface of the cut down to a depth of 100 μ was counted; the stage was then moved so that the adjoining field was brought into register and the particles counted as before; these operations were repeated along one traverse

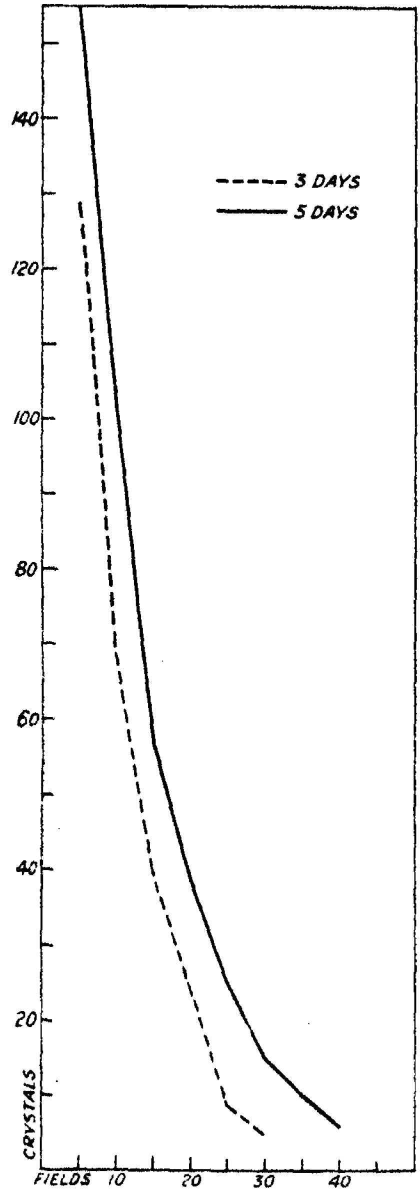

Fig. 6.—Curves showing the number of particles of indigotin produced by a colony of *Ps. indoloxidans* on the surface of indol agar in a petri dish after 3 days' and 5 days' growth.

The upper curve confirms the observation made, that more indigotin is produced near the bacterial growth. The lower curve suggests that crystals produced at some distance from the line of stab are due to the activity of the organisms on the surface. Apparently the organisms cannot initiate the process at the lower depth owing to insufficiency of oxygen, but once they have initiated it at the surface they can form crystals at this depth. This supports the hypothesis that the action is due to the production of an exo-enzyme diffusing out from the bacterial growth.

Production of Indigotin in Plate Cultures.

In plate cultures surface colonies (measuring about 0·5 mm. in diameter) were usually visible after 48 hours' incubation; deep colonies measured about 0·1 mm. long diameter. Indigotin was already visible around surface colonies, being distributed horizontally to a distance of at least 2·25 mm. from the edge of the colony. The largest particles measured 3 μ to 3·5 μ long, but the majority were too small to enable a count to be made with sufficient accuracy. The growth of the organism was stopped by exposing the plate to the vapour of formaldehyde; examination made at intervals for some days showed that production of indigotin had also ceased. The colonies on a

up to the edge of the agar. The section was then reversed and the particles counted at 50 μ to 100 μ below the surface of the lower cut. In drawing up the curves the number of particles of indigotin in every 5 fields was added together in order to smooth out differences due to inequalities of the gel.

plate culture not so treated continued to produce indigotin to the maximum distance. A count was made on a series of plates on which the production of indigotin took place between the forty-eighth and sixty-sixth hours. The colony chosen was 0·95 mm. in diameter, and three traverses along horizontal columns of agar each 50 μ thick were made in one direction to a distance of 3·3 mm. from the edge of the colony. The result of this count is shown by the broken line in fig. 6. Counts were made from this colony two days later (the colony having then grown to 1·2 mm. in diameter), in order to ascertain if any increased oxidation had taken place. The result is shown by the unbroken line, the points on which represent the mean of two traverses on opposite sides of the colony. The particles slightly increased in number between the third and the fifth day.

The shape of these curves approximates closely to that of a diffusion curve. This is what would be expected on the hypothesis that the process is effected by an exo-enzyme diffusing outwards from the bacterial growth. If, on the other hand, the indol were taken in by the organism and excreted as indoxyl the probability is that the oxidation of the latter, as soon as it reached an area of higher oxygen-tension outside the colony, would appear as a thick zone of blue particles close to the colony. This, however, does not take place at any stage of growth. Furthermore, colonies buried at the bottom of a 5-mm. thick layer of indol agar produced a similar curve for the vertical distribution of indigotin, the maximum amount being formed at the shortest radius from the colony (see fig. 7).

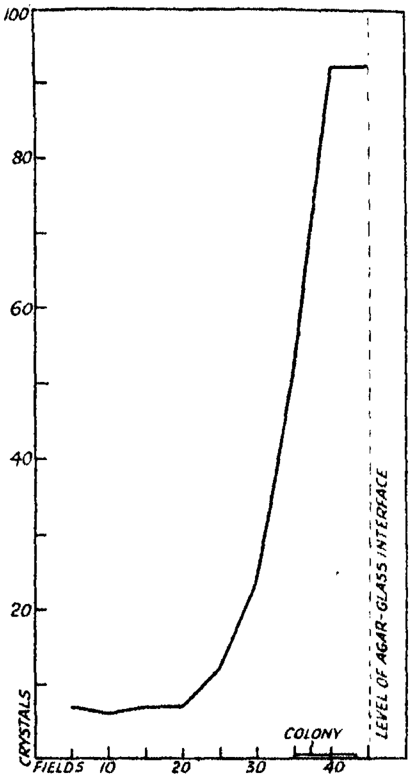

FIG. 7.—Curve showing the number of particles of indigotin produced by a colony of *Ps. indoloxidans* situated near the floor of the petri dish culture. Semi-diagrammatic.

The Carbon and Nitrogen Requirements of Ps. indoloxidans.

In these experiments the same mineral salt solution was used as in agar cultures. Except when amino-compounds were supplied, ammonium sulphate was generally given as the source of nitrogen, since the production of free nitric acid from nitrates might cause a loss of indigotin. Carbon was supplied by the compounds as shown in the experiments reported below. The cultures were usually incubated at 22° C. to 25° C.

Since the production of indigotin appeared to be associated with the active growth of the organism, an experiment was carried out to correlate, if possible, the growth in number with the production of indigotin in solution cultures. Spindle-shaped flasks of 300 c.c. capacity containing 100 c.c. of solution with glycerol 0·1 per cent. and $(NH_4)_2SO_4$ 0·05 per cent. and 10 mgms. of indol were inoculated each with about 20 millions of cells per flask and incubated for 9 days

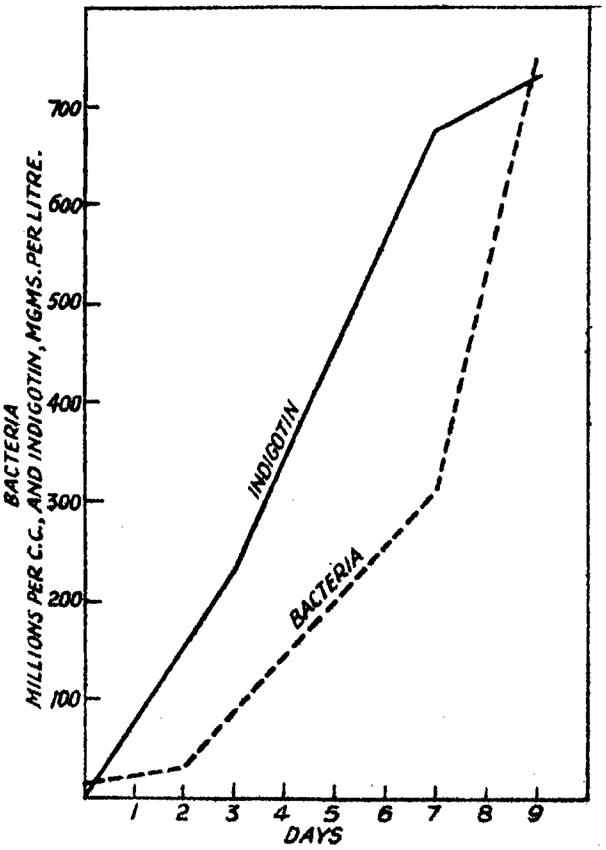

FIG. 8.—Curves showing bacterial numbers and amount of indigotin produced.

at 25° C. A blue tinge due to the indigotin was visible on the second day, when a hæmocytometer count of the total bacterial cells showed 30 millions per cubic centimetre.

The result of the analyses for indigotin* and the bacterial counts are shown in fig. 8. Indol was present, as shown by the pink colour given with Ehrlich's dimethyl-*para*-amino-benzaldehyde reagent, on the third and seventh day, but had disappeared on the ninth day. The production of indigotin is associated with rising bacterial numbers; the falling off on the ninth day is associated with the disappearance of the indol.

It seemed possible that this depression might be due to the organism using indol as a source either of energy or of nitrogen. In order to test this, flasks of basal media containing the compounds as given in Table I were inoculated and incubated for 3 days.

Table I.

	Additional nitrogen.			
	$(NH_4)_2SO_4$.		Nil.	
	Growth.	Indigotin.	Growth.	Indigotin.
Carbon compound—				
1. Indol	0	0	0	0
2. Glycerol	+	0	0	0
3. Tryptophan	+	0	+	0
4. Glycerol + tryptophan	+	0	+	0
5. Glycerol + indol	+	+	+	+
6. Tryptophan + indol	+	+	+	+

From this experiment it was concluded that under these conditions the organism—

(*a*) cannot use indol as a source of energy;

(*b*) cannot oxidise indol to indigotin without an additional source of carbon;

(*c*) can oxidise indol (given a supply of carbon) without an additional supply of combined nitrogen, and thus probably obtains nitrogen from the indol.

An analysis of the medium, without indol or ammonium sulphate, showed that the amount of nitrogen present was only 0·0000025 per cent., which is considerably less than the analytical error.†

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* The method used for estimating the amount of indigotin was essentially Rawson's, as described by W. A. Davis in 'Agric. Res. Inst. Pusa' (Indigo Publications), 1921.

† The error was about 0·000025 per cent. I am indebted to Dr. H. L. Richardson for this estimation by the Kjeldahl method.

It is interesting to note from Table I that the organism cannot convert tryptophan into indigotin; neither indol nor scatol was at any time produced. Scatol is not attacked by the organism.

A high C : N ratio appears to be most suitable to the growth of *Ps. indoloxidans* and the production of indigotin. The following amounts of glycerol and ammonium sulphate were added to 100 c.c. of the mineral salt solution :—

	Glycerol.	$(NH_4)_2SO_4$	Ratio.	
			C	N
	gm.	gm.		
Culture *a*	0·5	0·0126	16	1
,, *b*	0·125	0·0126	4	1
,, *c*	0·031	0·0126	1	1

The above quantities were added from sterile solutions to the autoclaved mineral salt solution containing the indol. After 8 days' incubation at 25° C., indol still

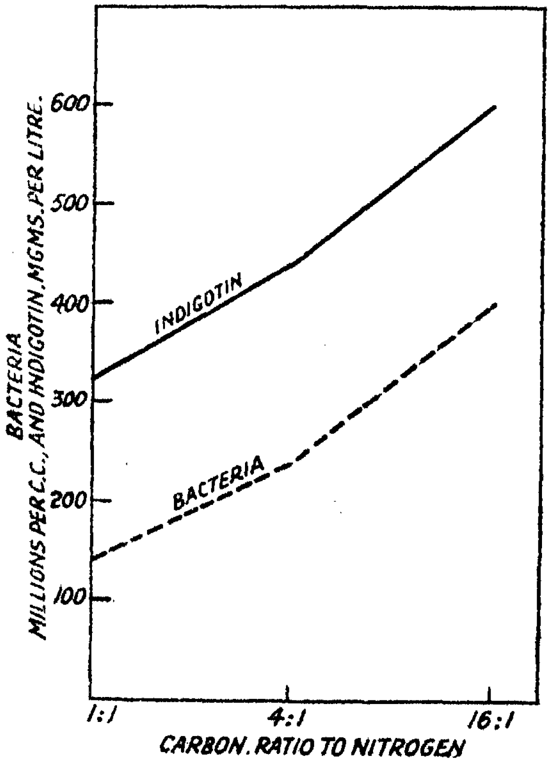

Fig. 9.—Curves showing the relation between bacterial numbers and amount of indigotin produced at different ratios of carbon to nitrogen.

occurred in flask (c), but not in the other two. The total bacteria and the amount of indigotin produced are shown in fig. 9. The bacterial numbers and the amount of indigotin produced both increase with higher ratios of carbon to nitrogen.

Availability of Organic Compounds.

In order to discover the relative value of different carbon compounds as sources of energy, flasks of media containing ammonium sulphate as the additional source of nitrogen, were set up with the carbon compounds shown in Table II in the concentration 0·1 per cent. (except the media containing no additional carbon compound, that with cellulose, and that with naphthalene). The concentration of indol was 0·01 per cent. The cloudiness or otherwise of the solution and the time when the blue of the indigotin was first noted are indicated.

Table II.—Relative Availability of the Different Sources of Energy.

A—The following compounds did not supply energy to the organism and indigotin was **not** produced:—

Methyl alcohol, amyl, capryl, formic, sodium formate, calcium formate, ammonium oxalate, valerianic acid (normal), xylose, sucrose, cellulose, phenol, naphthalene, indol.

B—Compounds supplying energy to the organism.

Indigotin produced.			
Solution clear. (Growth slight.)	Blue first noted.	Solution cloudy.	Blue first noted.
	Days		Days.
		Ethyl alcohol	2
		Propyl alcohol, normal	5
		Propyl alcohol, iso	5
		Butyl alcohol, normal	3
		Butyl alcohol, iso	3
		Acetic acid	3
		Propionic acid	3
		Butyric acid	3
		Butyric acid, iso	3
		Valerianic acid, iso	3
Adonitol	12	Glycerol	2
Dulcitol	14	Mannitol	5
Arabinose	13	Dextrose	3
Galactose	5	Lævulose	5
Lactose	13		
Maltose	13		
Raffinose	19		
Starch	20	Glycollic acid	3

Glycerol was thus the most suitable of the compounds tested for supplying the organism with an easily assimilable source of energy. It is of interest to note that the organism behaves differently towards closely related compounds, *e.g.*, xylose and arabinose, and the *normal* and *iso* forms of valerianic acid.

The relative value of certain amino-compounds as energy sources was also tested. In this case, the ammonium sulphate was omitted from the solutions and no additional source of energy was supplied.

Table III.—Relative Value of certain Amino-Compounds as Sources of Energy to *Ps. indoloxidans*.

No indigotin produced.	Indigotin produced from indol with	Blue first noted.
		Days.
Acetamide	Urea	8
	Asparagine	4
	Glycine	4
Cystine	Alanine	4
Valine	Tyrosine	8
	Tryptophan	1
o-Anthranilic acid	Aspartic acid	1
Isatin		

Growth was good on all of the media in which the indigotin was produced, but of the other compounds cystine alone supported any growth.

Purity of the Indigotin.

The indigotin as collected on the asbestos filter bed previous to sulphonation is mixed with a considerable amount of impurity, *e.g.*, mineral salts precipitated in the medium and bacterial growth. The amount of crude indigotin collected from cultures in which the bacteria had completed or passed their period of maximum growth is a fairly constant one, about 20 to 35 milligrams per 100 c.c. of culture. The presence of these impurities may affect the curve obtained by spectro-analysis, especially in the visible region. The following are a few of the results obtained from the analysis of about 40 cultures.

The figures in the final column represent the amount of indigotin produced expressed as a percentage of the amount of indol supplied. Since, however, 112 gms. of indigotin may be produced from a 100 gms. of indol, the percentage figures must be divided by 1·12 to arrive at the percentage of efficiency of the oxidation.

The cultures quoted in Table IV, with the exception of No. 5, were grown at 23° C. to 25° C., but were not parallel cultures. No. 5 consisted of 500 c.c. medium in a Woulff's bottle, and was grown with a current of air flowing through continuously for the time stated at 10° C. to 12° C. (room temperature).

Table IV.—Indigotin produced by Cultures of *Ps. indoloxidans* in Solutions with various Sources of Energy and Nitrogen.

Carbon.	Nitrogen.	Age.	Crude indigotin.	Pure indigotin per cent. of crude.	Indigotin per cent. of indol supplied.
			mgms.		
1. Glycerol	$(NH_4)_2SO_4$	3	9·0	28·6	25·7
2. ,,	,, ...	16	10·5	60·7	63·7
3. ,,	,, ...	19	21·0	39·7	63·3
4. ,,	a-amino propionic acid	18	24·9	30·2	75·3
5. ,,	asparagine, KNO_3 ...	10	140·5	33·5	94·1
6. Amino-acetic acid		9	24·0	27·6	66·2

Efficiency of Oxidation.

In the experiments quoted above the best conditions for a maximum yield of indigotin may not have been found. While the indol can supply nitrogen and thus reduce the amount of indigotin produced, some of the indol may have been lost by volatilisation during sterilisation. An experiment was therefore set up, having the indol melted in the glycerol ammonium sulphate medium after the latter had been sterilised. The inoculated flasks were incubated at 25° C. for 4 days, when the bacteria in 1 c.c. from each flask were counted by plating on nutrient agar, and the remainder of the culture filtered to collect the indigotin. The result was as follows :—

	Bacteria, millions per c.c.	Indigotin, mgms.
Culture 1	268	11·39
,, 2	298	9·17

The first of these two cultures gave the highest percentage conversion (100 per cent.) of the indol that has been obtained. The efficiency of oxidation can, therefore, be raised by this procedure.

Toxicity of Indol.

Indol appears to depress the growth of the organism. This was tested by growing the organism on media containing the usual amount (0·01 per cent.) of indol, and half of that concentration. A comparison was made at the same time between glycerol and dextrose as sources of energy. The bacteria were counted by plating on nutrient agar after 4 days' incubation at 25° C. The result is shown below.

Growth of *Ps. indoloxidans* at Two Concentrations of Indol.

Indol. per cent.	Bacteria, millions per c.c. Dextrose.	Bacteria, millions per c.c. Glycerol.
0·005	327	237
0·01	22·5	25

The higher concentration of the indol therefore prevents rapid growth of the organism. It was also noted from this and other experiments carried out under similar conditions that the organisms multiplied rapidly as soon as the indol had been used up.

Delayed Addition of Indol.

The effect of adding indol to a culture after growth had started was also tested. Flasks of mineral salt solution of the usual composition, with glycerol, were inoculated and incubated at 27° C. After 2, 4 and 7 days' growth 10 c.c. of a 0·1 per cent. solution of indol was run into the cultures. Blue crystals appeared within 24 hours in the cultures thus treated after 2 days, but not in those treated after 4 and 7 days' growth. The production of indigotin, however, after 2 days' growth, was not continued to any extent. It appears, therefore, that the oxidation of indol takes place only during the early stages of growth of the organism.

Intermediate Products.

If the conversion of indol to indigotin by *Ps. indoloxidans* is a simple oxidation, indoxyl would be expected to occur as an intermediate product. It has, however, been suggested (see pp. 268–269) that it is improbable that the excretion of indoxyl takes place from within the cells of *Ps. indoloxidans*. Davis* states that the presence of unoxidised indoxyl in the liquor of the beating vat may be detected by exposing a little of the solution on filter paper to the fumes of ammonia; if free indoxyl is present the paper becomes blue or green. No trace of such a reaction has been found in several cultures of *Ps. indoloxidans*, all of which remained alkaline; traces of indoxyl would not remain in solution long enough to allow detection by that method. From the further fact that the whole of the indol supplied can be oxidised to indigotin, it also seems unlikely that the presence of free indoxyl could be demonstrated.

Further Oxidation Products.

Since indigotin may be further oxidised to compounds which are soluble in water, and therefore not collected in the precipitate, an attempt was made to discover the presence of isatin in older cultures. The method adopted to test for the presence of isatin was that given by Porcher.* This consists in acidifying the solution to be tested and shaking it up with ether ; the ether extract is evaporated on a porcelain dish and the residue treated with a few drops of benzol containing 1 per cent. thiophen, when the presence of isatin is indicated by a bright blue rim. No trace of this reaction has been observed in several cultures on which this test was made. It has already been stated that loss of indol by volatility may account for the yield of indigotin being less than that which it is theoretically possible to obtain, and that this loss can be overcome by omitting sterilisation. It seems improbable, therefore, that indigotin is further oxidised to isatin by this organism.

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* 'Agric. Res. Inst. Pusa' (Indigo Publications), 1921.

Summary and Abstract.

1. A soil organism, *Pseudomonas indoloxidans*, n.sp., oxidises indol to indigotin in solution cultures and on agar media.

2. The indigotin is formed as crystals outside the organism, appearing first within 20 hours on agar media. The crystals decrease in number with increasing distance from the bacterial growth.

3. Indol does not act as a source of energy, some other source being necessary to effect the oxidation of indol to indigotin. Of a number of carbon compounds tested, glycerol appears most readily to act as a source of energy in the oxidation. Bacterial numbers and the amount of indigotin produced increase with higher ratios of carbon to nitrogen. The organism can utilise trytophan as an energy source but produces neither indol nor indigotin therefrom.

4. Indol is oxidised only by young growing cultures, and can be oxidised in the absence of other nitrogen compounds ; it depresses the multiplication of the bacteria.

5. No trace of indoxyl has been found in cultures, and the indigotin is not further oxidised to isatin.

6. Two other soil organisms, *Mycobacterium globerulum* n.sp. and *Micrococcus piltonensis* (Gray and Thornton) n.sp., can also produce small amounts of indigotin on indol agar only.

I wish to thank Mr. H. G. Thornton, Head of the Department of Bacteriology, for his unfailing interest and valuable suggestions.

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* 'Bull. Soc. Chim. de France' (IV), vol. 5, p. 526 (1909).

DESCRIPTION OF PLATE 21.

FIG. 1.—*Mycobacterium globerulum*, showing capsules stained by Muir's method. From a camera lucida sketch. (× 1100.)

FIG. 2.—*Mycobacterium globerulum*, showing some of the cells, stained with erythrosin, surrounded by indigotin. From a camera lucida sketch. (× 1100.)

FIG. 3.—Two crystalline particles of indigotin produced by *Pseudomonas indoloxidans*.

FIG. 4.—Photomicrograph of the edge of a surface colony of *Pseudomonas indoloxidans* on indol agar, showing particles of indigotin on the surface of the agar outside the colony.

FIG. 5.—The same, at a deeper focus, showing the hour-glass shaped particles below the surface.

A Consideration of Some Attempts to Analyse Growth Curves.

By G. E. Briggs, St. John's College, Cambridge.

(Communicated by Dr. F. F. Blackman, F.R.S.—Received November 9, 1927.)

No matter what attribute of an organism is considered—height, weight, activity, etc.—the rate of change of this attribute, the growth, depends upon the age of the organism. In many cases the rate of change is small at first, becomes faster and faster for a certain time, and then slower and slower. This drift of the rate of growth with increasing age is found when organisms are kept in constant environments. Such a drift in a constant environment may be taken as an expression of a drift in the organism itself, and the rate of growth can be taken as a measure of this "internal factor." If two precisely similar organisms were placed at their beginnings in two different constant environments, it is highly probable that the form of the drift of growth rate with time would be different. One of the organisms would be older than the other when a certain growth rate was attained.

Although we might say that at these points the intensity of the "internal factor," or the effective amount of "growing material" as it has been called (6), of the two organisms was the same, we do not mean that the organisms are identical. The "internal factor" determining the rate of growth is a kind of integration of all the various factors inside the organism. For example, in the case of the growth in weight of a plant, number and size of growing points, accessibility of food supplies, etc., are factors determining the dimensions of the "internal factor." The two organisms may differ in each of their subsidiary factors whilst possessing "internal factors" of like dimensions.

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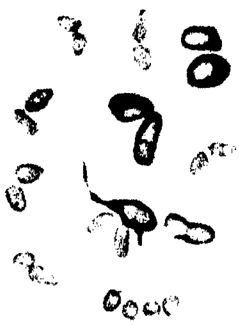

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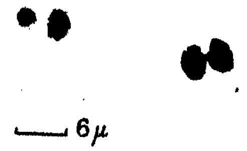

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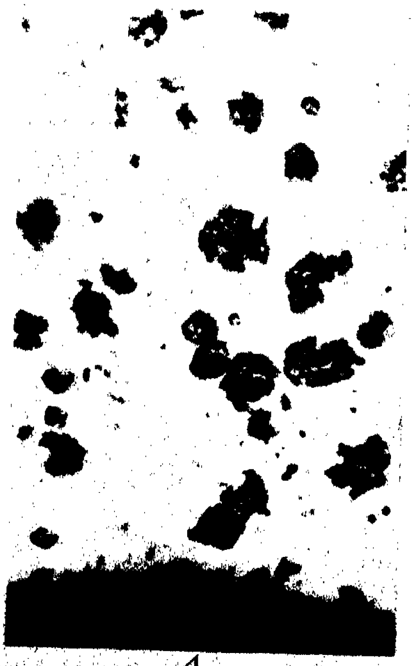

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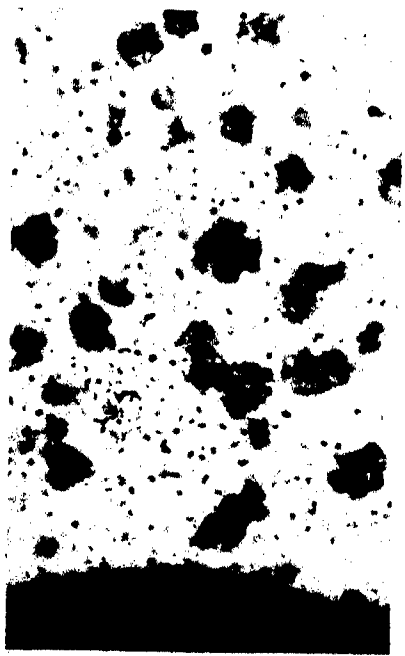

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(*Facing p.* 280.)

The rate of growth depends, not only upon the internal complex, but also upon the external complex which is called the environment. This is admittedly a crude division, for in many cases it is difficult to say whether a factor is to be included in the external complex or the internal, but it is sufficient for our present purposes. Two totally different environments might have the same effect on the growth rate of an organism, and from one point of view they could be considered the same. As we can usually analyse the environment, it is customary to consider separate attributes of the environment whenever possible. In many cases further analysis than at present achieved is desirable. For example, in the study of the growth of plants in relation to radiation, it is desirable to know something about its composition as well as its total intensity, the attribute usually and most easily measured. Perhaps in the future it will be possible to analyse the "internal factor" into growing material and "internal environment."

We can say then that the rate of growth of an organism is a function of the "internal factor," at present unanalysed, and the various attributes of the environment. Change of environment need not always have the same effect on the rate of growth. Even though two organisms have the same rate of growth under one set of conditions, yet the component parts of the "internal factor" may be different in the two cases and may be differently affected by change of environment.

The problems of growth are many, but one which has received some attention in the past is the evaluation of the effect of environmental conditions on the growth of plants under natural conditions. It is a consideration of some of these attempts which we propose to make. Although the experiments have been carried out with plants, the criticism is fundamental to all such attempts to analyse growth—whether of plant or animal individuals, living or non-living aggregates.

The data are as follows: (*a*) Rates of growth for definite combinations of "internal factor" and environment, and (*b*) various attributes of the environment. In the case of the constant environment we observe the *drift* of "internal factor." If we had organisms with identical "internal factors," identical in the values of the component factors, in environments which differed in but one attribute, we could ascertain the *relation* between rate of growth and dimensions of this attribute. But to determine both the drift and the relation is the problem.

Various attempts have been made to find a solution to this problem (1, 3, 5), and the impression that a method has been found seems to be gaining ground (4).

For in the latter paper we find "plants being the playthings of the changing external *milieu*, it is difficult to discern the laws which are fundamental for their growth . . . however, the influence of external variations on observations made on growth may be eliminated by calculations of correlation coefficients for different external factors and then obtaining normal values." With our present limited knowledge it would be too much to expect more than an approximate solution in the first place. Our first step must necessarily be tentative. The solutions we are about to consider are, in our opinion, not only tentative but are based upon an assumption for which no justification has been advanced and which in many cases is invalid.

We propose to discuss not the conclusions drawn from the analyses but the analyses themselves. Brenchley (1), having collected data of the growth rate of plants and the conditions of the environment, said let R_n, the growth rate at the *n*th unit of time from a certain zero time, be related to the attributes a_n, b_n, c_n,* etc., of the environment during the *n*th period of time by the following expression

$$R_n = A + Bn + Ca_n + Db_n + Ec_n + \ldots,$$

and by the solution of sets of equations she obtained best value for the constants A, B, C, etc. Whatever the terminology she used, the impression at least one reader derived from the paper is that its author thought that the rate of growth was determined by the age of the plant *measured in units of time* (A + B*n*) and the intensity of the external factors as expressed in the above equation.

Vyvyan's method (5) is made clear by the following statements: "The object of the methods . . . is to estimate the magnitude of this 'daily factor' on the successive days of the experiment in order that its influence may be eliminated and the 'ideal' values of the rate of growth ascertained. By 'daily factor' is meant the deviation of the observed growth magnitude on a date from the 'ideal' value that would have been obtained on that date had environmental conditions remained constant at their mean for the whole period of the experiment."

Gregory (3) substitutes the term "normal" for "ideal" and says: "If the shape of the normal curve (the normal values plotted against time) were known . . . the effect of changes in environment might be measured by comparing the slopes of comparable portions of experimental and normal curves." This is somewhat vague, but the procedure adopted is clear and was as follows: An approximation to a normal curve was obtained and the difference between the rate of growth calculated over the period between x per cent. and y per cent. of the time taken to attain the maximum size from the observed curve, and the rate over a similar period from the normal curve was taken to be due to the effect of external factors and was assumed to be related to them in the same manner as assumed by Brenchley. The chief difference is that one uses time in days and the other time in fractions of time taken to reach the maximum size.

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* These values are measured from the mean value.

Let us consider the assumptions made. They all assume that r_n being the rate of growth which the organism would have had in its nth period of existence if (a) the external conditions of all the preceding duration of its existence had been at the mean value, and (b) the conditions were still at the mean during the nth period, then the actual rate R_n will be related to r_n and e_n the value of the environment of the nth period by the following expression $R_n = f(r_n, e_n)$ which would be r_n if e_n had the mean value. Vyvyan tries two different forms for this expression, but eventually decides upon the form used by the other two investigators, that some function of the environment has to be added to the normal value r_n to give the observed value. With the justification of this portion of the procedure we are not here concerned.

The point is that these methods attribute the difference in rate of growth in the nth period of existence (measured in days or as fraction of the total existence) solely to the difference in the environment of that period. Since in all three the relative rate, that is, percentage increase and not the absolute increase, is used, the methods assume that in the nth period of existence the absolute rate of increase will be proportional to the size of the organism (its dry weight or whatever attribute is being measured). In other words, the difference in size is assumed to be a measure of the difference in "internal factor" determining the difference in the absolute rate of increase of size. In some simple cases, such as the size of a colony of unicellular organisms, such as yeast, in the exponential phase of growth, this may be so; in the exponential phase the percentage rate of increase does not change with time. But in the case of organisms such as the higher plants there is no evidence of which we know that justifies in the slightest degree such an assumption. In fact, the evidence is against it. Plants which are backward through previous experience often tend to catch up more forward plants of the same age when put under the same conditions as the latter. The same often applies to animals. In any case to retain the same degree of inferiority* for the rest of the life is unusual; inferiority perhaps, but not precisely the same degree.

---

* If forward and backward organisms have the same relative rate of growth the ratio of the sizes will not change.

It seems more likely that if the environment during the $n - 1$ previous periods had been below the mean, the rate on the nth period would have been r_{n-x} (the "normal" rate for some previous period) if the environment was mean for the nth period, or $f\ (r_{n-x},\ e_n)$ if the environment for the nth period had deviated from the mean. Or we can put the matter in another way and say that the actual rate is a function of (*a*) the "normal" value r_n, (*b*) the environment at the time, and (*c*) the previous environment, $R_n = f\ (r_n,\ e_1,\ e_2,\ \ldots\ e_n)$ for R_n is a function of the rate which would have obtained if the environment during the nth period had been average (ρ_n) and of the deviation of the environment of this period from the average, and this ρ_n is a function of r_n and the environment in the previous $n - 1$ periods.

The above methods involve another assumption for which no justification is attempted, and that is that a difference in the environment has the same effect on the relative rate of growth at all stages of the organism. Again, evidence is against the validity of the assumption.

We will consider the case of an autocatalytic reaction as a concrete example. This is not irrelevant, for although no attempt to apply the above methods to an analysis of such reactions has been made (so far as we are aware) yet such reactions may occur under natural conditions, and someone may apply a similar method of analysis in the future; if they have not already done so. Moreover, there are biologists who believe that growth and autocatalytic reactions are closely related. The course of such a reaction is expressed by the equation $R = Kx\ (A - x)$ where R is the rate at which the substance disappears, x its concentration and A its original concentration, K is a function of the environment. The relative rate, R/x at any moment will depend not only upon the environment but also upon $A - x$; the latter quantity depends upon the previous history. We are not suggesting that there is any fundamental relation between growth of organisms and autocatalytic reactions.

Another way of viewing the matter is as follows. In the methods considered the relative rate of growth is assumed to be determined by the "normal" rate for that time, this "normal" rate depends upon the average environment for the whole life. It is also determined by the deviation of the environment of that time from the average for the whole period, before and after. As it is possible to have the same average conditions made up of different previous and subsequent sets of conditions, the methods would be justifiable if subsequent environment as well as previous helped to determine the growth rate. In Gregory's method this may be so, since he uses time as a fraction of total time to reach the maximum size and this fraction depends upon subsequent environment. But despite this substitution of time as a fraction of the total for time in days, there is no evidence that when an organism has lived a half, or any other fraction, of its total span of life the relative rate of growth at that stage will be determined by the average environment for the whole life, no matter what the environment has been during the first half of the period. On the other hand, it seems likely that it will be dependent upon the environment for the first half of the period.

As a result of the above considerations, we suggest that the problem of the analysis of an observed growth rate into that part which we may call " normal " and that part which we may consider due to the deviation of the environment from mean conditions has not been solved by the above methods, and hence any conclusions based on such methods may be wrong.

It is to be expected that different lines of attack will have to be adopted for different growth problems. The method will depend not only upon the type of organism but upon our knowledge of internal processes and structure, etc., the factors determining the " internal factor." In the case of higher plants we have some knowledge of photosynthesis, the process by which the bulk of the dry material in the plant is produced. In another place Briggs, Kidd and West (2) suggested that for some plants during an appreciable portion of the life cycle the leaf area may be a measure of the amount of growing material, so far as increase of dry-weight is concerned. If that is the case the rate of increase of dry weight per unit leaf area, called unit leaf rate, should fluctuate about a mean, the fluctuations being determined by the fluctuations of the environment. If we could ascertain the form of the relation between deviation of unit leaf rate and deviation of environment then we should be a step forward in our analysis.

In conclusion, it may be said that this note is not intended to be a detailed exposition of the problems involved in the analysis of growth of organisms, but just enough to make clear why the writer believes that the methods criticised are not steps in the right direction for a better understanding of the drift of growth rate with time, and of the effect of environment on growth.

The writer is indebted to Dr. F. F. Blackman for reading the manuscript and for suggestions.

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Triplet Children in Great Britain and Ireland.

By R. A. Fisher, Sc.D., Rothamsted Experimental Station, Harpenden

(Communicated by Sir Arthur Keith, F.R.S.—Received October 25, 1927.)

Contents.

1. Origin of Material 286
2. Survival 288
3. Average Size 290
4. Precision of Measurements 293
5. Degrees of Resemblance 294
6. Inheritance of Favouring Tendencies........ 301
7. Summary 309

1. *Origin of Material.*

The study of twins is somewhat impeded in Great Britain by the absence of the registration of multiple births; for, contrary to the practice of most civilised countries, the occurrence of twins appears in the registers as two distinct births, occurring to the same parents at an unusually short interval of time. Consequently official data are lacking as to the frequency and sex distribution of twins and triplets. On the other hand, the existence of a Royal Bounty for triplets supplies a potential source of information, of which it is believed scientific use has not previously been made. The records of the Royal Bounty, in the charge of the Secretary of His Majesty's Privy Purse, constitute in effect a special registry of triplet births, including the great majority of the cases in Great Britain and Ireland. With the support of the Medical Research Council the author was kindly allowed access to this material, for which he desires to express his indebtedness to all concerned.

Visits to individual families for the purpose of measuring the surviving children made the enquiry a more expensive one than if the information had been gathered merely by correspondence. During three years the British Association made grants to a Committee, appointed by Section H, under the chairmanship of Dr. Shrubsall, in aid of this work, which together with a generous donation from Major Leonard Darwin covered nearly the whole of the actual expenditure. This would have been much greater had not a number of gentlemen (see below, p. 288), situated in districts distant from London, freely given their assistance in taking measurements.

A study of the conclusions arrived at in different investigations of twin births reveals two serious disabilities from which existing bodies of data ordinarily suffer. The most important collections of twin measurements, such as those of Thorndike (10), Dahlberg (3), and Lauterbach (4), refer to twins of ages from childhood to adolescence, with a few adult cases. Statistical allowance for age is not easy over a period which includes a well-marked maximum of growth rate, which occurs at considerably different ages for boys and for girls, and doubtless at different ages also for children of the same sex. Attempts have been made to eliminate the effects of varying age by comparison with a larger number of children from single births (10), data which are seldom available, and by the use of empirical growth curves based upon the twin measurements (4), a procedure which is useful only when the data are exceptionally extensive. Both methods, however, are open to the objection that possibly hereditary factors play an important part in the determination of precocity or the reverse, and that, if so, similar genetic variation in children of opposite sex may produce a materially less degree of resemblance between twins (who, of course, are usually at the same age at the time of measurement) than in the case of twins of like sex.

The second disability also affects collections of measurements, but in an even more serious degree appears to influence studies of the inheritance of the twinning tendency. It is that cases are brought to the notice of the investigator on account of their interesting character. The investigation may even have been initiated by some such interesting group of cases, and even if subsequent cases are collected by some sound method which excludes the possibility of unconscious selection, the collection will still in some degree retain its atypical character. Large collections, if not composite, escape this difficulty better than small collections, but with the increased precision with which tests of statistical significance are now carried out, it becomes the more important to emphasise that only data obtained on a carefully planned and uniform basis are capable under such tests of yielding valid conclusions. How far this consideration is capable of explaining the great discrepancies, not only between the conclusions of different investigators, but between the bodies of data they present, can only be known as material free from this suspicion is accumulated.

The material for the present study comprises three consecutive years of the King's Bounty records, with dates of birth ranging from October 8, 1917, to September 28, 1920. The period was chosen so that from the time the investigation was commenced surviving children should be available for measurement at a fixed age, which was selected in advance at 6½ years. If two or more triplet children survived to this age, they were measured. Since the limiting factor of the enquiry was the cost of visiting the children at this time, no attempt was made to recover at a later date the measurements of cases, which for one reason or another could not be measured at the right time. It would have been scarcely possible to obtain the measurements but for the assistance of Mr. E. M. Somerfield in Ireland, Dr. Alexander Low in Scotland, and Mr. L. H. C. Tippett who measured a large number of cases in the Midlands and North of England. Mr. A. R. Stoney Smith also measured several of the earlier English cases. To the care of these voluntary assistants in procuring accurate and comparable measurements must be ascribed the clear and unambiguous results to which the measurements data point.

All parents, whether the triplet children survived or not, were asked to fill in a relationship form, giving the numbers and sexes of all children, whether by single or multiple births, in certain nearly related families. The importance of obtaining complete data over a uniform range of kinship cannot be overemphasised. In many families remarkable cases of multiple births from comparatively remote relatives are thrust under the notice of the investigator, but must be ignored, since it is only for the families of near relatives of the father and mother of the triplets that complete and reliable information can ordinarily be obtained. The children born to the father and mother of the triplets and the children born to their parents are normally well known; in addition, the questionnaire covered the children of the brothers and sisters of the parents of the triplets. The latter contain a proportion of incomplete families, and a number of individual families had to be omitted owing to migration. Only cases in which the number and sexes of the children could be listed with confidence were included.

The numerical data, together with the specification of the measurements taken, have been filed at the Natural History Museum at Sonth Kensington, under the arrangements made for the conservation of biological measurements by the Measurements Committee of the British Association.

2. *Survival.*

In all 166 cases of triplet births were available for enquiry; of these, however, no reply could be obtained in 20 cases, either from change of address since the birth of the children, or more rarely from the parents being unwilling to supply information. The data for survival in the remaining 146 cases are given below.

Table I.—Survival of Triplet Children Born Alive.

Born.	3 boys.	2 boys, 1 girl.	1 boy. 2 girls.	3 girls.	Total.
All living	2	4	8	6	20
One dead—					
Boy	7	4	10	—	21
Girl	—	3	12	6	21
One living—					
Boy	6	8	3	—	17
Girl	—	1	11	9	21
Sex unknown	—	2	2	—	4
All dead	7	15	14	6	42
Unknown	8	3	5	4	20
Total	30	40	65	31	166

The proportion surviving differs in the two sexes, but does not seem to differ greatly in the four types of triplet birth. From the table above the following percentages of survival are calculated :—

Tabls II.—Percentages Surviving.

	3 boys.	2 boys, 1 girl.	1 boy, 2 girls.	3 girls.	Total.
Boys	39	37	40	—	39
Girls	—	26	51	48	46

The low proportion of survival among the girls, in sets with two boys and one girl, is noticeable, but this is based on only 35 cases, of which 9 survived.

As is to be expected both from the genetical similarity of the children and from the similarity of their experience up to and including birth, the deaths of one or more children in the same set are far from independent. Of 101 pairs of two boys, in 20 cases both survived, in 38 one survived, and in 43 cases neither survived ; thus the chance of survival of a boy is raised to 51 per cent. if his brother lives from the value 31 per cent. if his brother dies. Of 188 pairs of opposite sex, in 40 cases both lived, in 32 cases the boy only survived, in 37 cases the girl only survived, and in 79 cases both died ; thus the chance of survival of the boy of such a pair is raised from 29 per cent. to 52 per cent. by the survival of his sister ; while the chance of survival of the girl is raised from 32 per cent. to 56 per cent. by the survival of her brother. Finally, of 140 pairs of two girls, in 42 cases both survived, in 53 cases only one survived, and in 45 cases neither survived ; consequently the chance of survival of a girl is raised from 37 per cent. to 61 per cent. by the survival of her sister. The association seems to be just as close between children of opposite sex as between children of the same sex, a fact which suggests that, with the high mortality experienced by triplet children, genetic similarity has little to do with the similarity of the chances of life.

3. *Average Size of Triplet Children.*

The children actually measured numbered 49 boys and 68 girls. The averages of the six measurements taken for each type of triplet births and for the aggregate of all types are given in Tables III and IV.

Table III.—Average Size of Boys.

Type.	Number of individuals.	Stature.	Stem.	Span.	Left cubit.	Head.	
						Length.	Breadth.
		mm.	mm.	mm.	mm.	mm.	mm.
3 boys	18	1102	624	1090	292·0	178·3	143·4
2 boys, 1 girl	16	1127	640	1128	304·5	179·3	140·8
1 boy, 2 girls	15	1092	624	1100	290·8	176·3	140·2
Aggregate	49	1107·0	629·2	1105·6	295·7	178·0	141·6

Table IV.—Average Size of Girls.

Type.	Number of individuals.	Stature.	Stem.	Span.	Left cubit.	Head.	
						Length.	Breadth.
		mm.	mm.	mm.	mm.	mm.	mm.
2 boys, 1 girl	8	1106	628	1084	292·9	170·5	138·3
1 boy, 2 girls	38	1078	610	1071	283·9	172·2	135·5
3 girls	22	1065	608	1060	278·5	168·5	136·6
Aggregate	68	1077·0	616·0	1069·1	283·3	170·8	136·2

It would be of great interest to compare the general averages with those of children by single births of the same age. The growth curve of children is, however, only known for a few special districts, and no general figure is available for the whole country, such as would be comparable with the triplet material. Dunstan (2) gives valuable figures based on 300 measurements of stature in each trimester for the children of the rural area of E. Sussex. At 6½ years of age his boys averaged 1114·4 mm. and his girls 1103·2 mm. Comparison may also be made with the average heights obtained from Miss Elderton's distribution for Glasgow School children (1), Table III, p. 296.

Table V.—Average Stature of Children at 6½ Years.

	Triplets.	E. Sussex, rural.	Glasgow A, poorest district.	Glasgow B, poor district.	Glasgow C, better class.	Glasgow D, still higher class.
	mm.	mm.	mm.	mm.	mm.	mm.
Boys	1107·0	1114·4	1070·6	1093·4	1093·4	1115·0
Girls	1077·0	1103·2	1065·6	1088·4	1087·2	1111·2

The differences between the different districts, though not exceeding 2 inches, are sufficiently great to leave in some uncertainty the comparison of triplet with single children. They are, of course, far too great to be ascribed to random sampling, but neither environmental nor hereditary factors can be excluded. With boys the average stature at this age in E. Sussex agrees with the "still higher class" schools in Glasgow, and only these two give a higher average than the triplets; the triplet girls do not compare so well, being taller only than the Glasgow girls from the lowest class districts. The averages cannot be taken to indicate that triplet children are on the average less tall at 6½ years of age than are children from single births. The distinguishing feature of the triplet measurements is not their average values, but the large sex difference. In the collections based on large samples to which the comparison is made, the sex difference is about 11 mm. in E. Sussex and only about 5 mm. in all the Glasgow groups; the triplets show a difference of 30 mm. If standard errors are calculated for the triplet children regarded as independent samples, the values would be only 6 and 5 mm., and would not suffice to explain the large sex difference.

Actually, the children are drawn in batches of two or three from the same family, a circumstance which tends somewhat to increase the liability to error of the two averages; on the other hand, the fact that many of the pairs of opposite sex are brother and sister will tend to diminish the error of the difference. In order to obtain a strict test the sex difference was obtained for the 23 families in which both boys and girls had been measured, counting each family as only a single observation. The mean sex difference is then found to be 18 mm., with a standard error of nearly 9 mm.; there are, in fact, somewhat large differences between different families measured in the relative stature of the boys and the girls, but the remarkable sex difference observed in the stature of the triplets is largely due to the boys having come predominantly from the taller families.

The average stature of the two sexes, 1092 mm., is seen to correspond with districts B and C of Glasgow, being about three-quarters of an inch less than that of rural E. Sussex and Glasgow district D, and nearly an inch greater than Glasgow district A. This simple result is one of the most surprising of the enquiry. It is well known that the correlations observed between the measurements of near relatives are only intelligible on the view that at least 90 per cent. of the variance observed in adult stature is due to genetic differences (8). This conclusion does not exclude the induction by environmental causes of an increase or decrease of stature of the order of half an inch, as a frequent occurrence; nor does it exclude effects of greater magnitude induced by extraordinary circumstances. The pre-natal conditions of triplet children must be exceptionally unfavourable, and the birth is very frequently premature. Perhaps the best measure of the severity of the environment which they have experienced consists in the very large post-natal death-rate. The conditions are sufficiently severe to kill more than half the children, but appear to be almost, if not entirely, inoperative in checking the normal growth of the survivors.

It will not be argued that the recovery of these children to normal stature is due to their environmental conditions during infancy and early childhood being in any way above the average; on the contrary, since the wage system in this country makes no allowance for the burden of offspring, we may be certain that the very existence of the triplet children will ordinarily induce a condition of economic hardship, which will last at least as long as the children remain dependents. An equally important circumstance is that, apart from economic stress, the amount of the care and attention ordinarily bestowed by their mothers upon children at these ages must be much diminished. It would appear that we must conclude that whatever may be the case in animals with a shorter growth period, the long duration of infancy and childhood in man gives ample opportunity for full recovery from the effects of pre-natal conditions, and for the full realisation of the genetic potentialities, even in characters, such as general growth, in which environmental modification would seem to be most readily effected.

4. *Precision of Measurements.*

The precision attainable in different human measurements does not seem to have been at all fully studied. In an investigation like the present in which it is important to determine with accuracy the degree of resemblance between children, many of whom may be of monozygotic origin, it is essential not only that no effort should be spared to attain adequate accuracy, but that objective evidence of the accuracy actually attained should be put on record. It was therefore arranged that for the majority of the cases measured, and especially for those of which the author had the greatest suspicion (namely, his own) that not only should the measurer satisfy himself that an accurate measure had been taken, but also that independent duplicate measures of the same value should be obtained, in order to yield by subsequent comparison an independent estimate of the accuracy of measurement. In doing this the specific purpose of the accuracy aimed at was held in view. The differences between different families of children are comparatively large, and these differences are to be used only in estimating the general variance of triplet children of a given age. The small systematic errors which undoubtedly exist between the measures obtained by different observers using different instruments will be entirely unimportant in estimating this variance, which will, on the contrary, be seriously affected by paucity of material, and should, in the author's opinion, be obtained from such an accurate estimate of the variance of the population as could be made from a carefully planned anthropometric survey based on the sampling method. In the second place, the small fluctuations, which, on the analogy of laboratory determinations would doubtless affect the same observer making the same measurements on different days, have fortunately no relevance to the comparisons between the measurements of different children on the same day and in the same circumstances. The errors in such comparisons should, in fact, be perfectly represented by the discrepancies between duplicate measurements taken at the same sitting.

The variance of a single observation of each measurement is given in Table VI.

If two values for the same measurement showed a marked discrepancy, a third was taken, a procedure which served to exclude gross errors. All measurements left on record were used in the above table, each case contributing 1 or 2 to the number of comparisons according as 2 or 3 measurements were left on record. The standard error of the values adopted will be nearly that calculated from the mean of two measurements, though this will be a slight over-estimate

Table VI.—Variation in Reported Measurements.

	Number of comparisons.	Single measurements.		Mean of 2, standard error.	Per cent. of like-sex variance.
		Variance.	Standard error.		
		mm^2.	mm.	mm.	
Stature	77	6·171	2·48	1·89	0·57
Stem length	73	12·751	3·57	2·53	2·80
Span	76	40·964	6·40	4·53	3·15
Cubit	77	3·356	1·83	1·30	3·03
Head length	75	0·691	0·831	0·588	3·53
Head breadth	70	0·331	0·575	0·407	2·46

owing to the cases, which include measurements made in the least accurate circumstances, in which the mean of three values was employed. The last column expresses the variance ascribable to errors of measurement as a percentage of the variance ascribable to differences between triplets of like sex, calculated in a similar way. Since in no case this amounts to 4 per cent., it is evident that the estimate of degree of resemblance even between the like-sex pairs, will not be vitiated in the case of any one measurement by the working errors.

Dahlberg (3) gives a most valuable account of the errors of measurement detected in his study of the resemblance between twins. The values given, on p. 199 of his book, for 15 bodily measurements refer to the standard difference between single measurements, and are therefore double the standard errors of the mean of two measurements. For length of head, Dahlberg's figures indicate a higher precision than that attained in this enquiry, for the standard error of the mean of two of his measurements would be only 0·35; for breadth of head, his standard error, 0·415, is about the same as that given by our own data, but for stature his value is 2·69, a larger value than the 1·89 indicated above, though not one which would detract from the value of his observations.

5. *Degrees of Resemblance.*

5.1. *Resemblance of Triplets of Unlike Sex.*—The triplets living to be measured supply 34 cases of pairs of opposite sex. These must be regarded as dizygotic pairs. If they are also biovular, provided the fraction of the variance for which environmental variations is responsible is quite small, they should show the same degree of correlation as that shown by adult brothers and sisters by different births. This test was applied to Lauterbach's data, an attempt being made to eliminate the age of measurement by fitting a cubic growth curve to the measurements of each sex. The values then found were somewhat low (see Table VIII) compared to well-ascertained fraternal correlations from single births, with the exception of that for cephalic index, which in that body of material showed no appreciable differentiation due to age or sex. It might therefore be supposed that this procedure could not, in view of the marked spurt of growth at puberty, entirely eliminate the disturbance due to varying ages.

As appears from the table, the present material based on measurements at a fixed age does, in fact, yield higher values, comparable with that obtained for cephalic index in Lauterbach's data. The natural supposition that weight, which yields the lowest unlike-sex correlation, is in reality not so highly correlated, even in growing children, as are the skeletal measurements, is not supported by the high values found for this variate between like sex pairs. The difference between the averages of the two series is, therefore, in so far as it is not ascribable to random sampling, probably due to the uniform age of the triplets.

Table VII.—Covariation of Pairs of Opposite Sex.

	Mean.		Sum of squares.		Sum of products.	Correlation.	
	Boys.	Girls.	Boys.	Girls.		r.	z.
Stature	1104	1083	71,540	51,594	32,733	0·539	0·602
Stem length	630	622	17,473	8,984	51,123	0·409	0·435
Span	1111	1073	86,927	62,219	37,946	0·516	0·571
Cubit	296·4	286·5	12,206	8,926	7,032	0·645	0·767
Head length	176·8	170·3	1,899·0	1,059·7	670·0	0·472	0·513
Head breadth	140·0	136·4	1,084·1	709·4	573·4	0·654	0·782

Table VIII.—Correlations between Brother and Sister.

		r.	**Mean r.**
Single births	**Stature**	0·553	0·508 ± 0·012
	Span	0·525	
	Cubit	0·440	
Twins	**Stature**	0·455	0·458 ± 0·053
	Stem length	0·461	
	Weight	0·380	
	Cephalic index	0·537	
Triplets	**Stature**	0·539	0·545 ± 0·074
	Stem length	0·409	
	Span	0·516	
	Cubit	0·645	
	Head length	0·472	
	Head breadth	0·654	

In view of the sampling errors no significance should be attached to differences between the correlations of the different characters; comparison of the average correlations obtained from single births (7), twins (4), and triplets leaves no doubt that the correlations are substantially equal. Moreover, if any appreciable part of the correlation observed between brothers and sisters were not of genetic origin, but were due to the general similarity of the environment to which children of the same parents are exposed, the correlation should be appreciably higher with twins or triplets. Since this is not the case the data confirm the view that no appreciable proportion of the resemblance is due to similarity of environment.

It should be noted that the range of home environment in the case of triplets was extremely high. In not a few cases the support of the triplets, in addition to previous children, sufficed to lower the subsistence level to one of extreme hardship during the childhood of the triplets. Other cases were comparatively well-to-do. In somewhat less degree it is probable that the same is true of the American twins in Lauterbach's record. The point is one of some importance, since it may reasonably be argued that Pearson's data, predominantly from the families of University students, do not represent such extreme environments as would be sufficient to produce permanent retardation of growth; and that the conclusion drawn (8) by comparison of these values with expectation on the assumption of Mendelian inheritance, that no appreciable fraction of the variance can be ascribed to environmental differences, though true for the class sampled by Pearson, ought not to be extended to classes in which child life is attended by real and severe hardships. The data presented do not suggest appreciable environmental effects in any class for children born from 1917–1920; nevertheless, on a point of such importance it may be worth while to call attention to the possibility of making a crucial test of the matter with more precision than the present enquiry attempts, by consistently accumulating the evidence supplied by triplets, as in this country it is possible to do, taking as control an adequate number of children by single births of the same age, but of superior class.

5.2. *Resemblance between Triplets of Like Sex.*—The correlation between pairs of triplets of like sex may best be found by taking the mean measurements of two or more such children in the same way as have been treated the two or more measurements of a single child. In this way not only is the constant negative bias of intra-class correlations derived from a symmetrical table avoided, but no undue weighting is given to the sets of three as opposed to the sets of two; moreover, the accuracy of the determination corresponds to that of a number of simple pairs providing an equal number of comparisons. The measurements comprise six cases of all three children of the same sex living to be measured, and 32 pairs of the same sex, the material being thus equivalent in respect of the estimation of the variance within such a trio to 44 pairs.

The value thus obtained, for the variance within a trio, is shown below (Table IX), for the six characters measured. It is interesting to compare them with the results obtained by Dahlberg (3). Dahlberg expresses his results as a mean difference between pairs of twins; his figures have therefore been squared and multiplied by $\pi/4$. Mean values for boys and girls have been used, and since Dahlberg's material consists of persons of very different ages, the comparison may best be made on the basis of the average actual measurement at each age. The unit is thus one-thousandth of the mean measurement in each case. Comparisons are possible for three characters. By a careful examination of the facial resemblance, and especially of the character of the ears, Dahlberg has diagnosed his material as monozygotic and dizygotic respectively, ignoring for this purpose the measurements actually obtained. The comparison thus provides a rough means of estimating what percentage of the triplets of like sex are of these two types of origin, assuming the accuracy and comparability of Dahlberg's figures.

Table IX.—Like-Sex Variance within Twinship or Trio.

	Triplets.	Dahlberg dizygotic.	Triplets.	Dahlberg monozygotic.	Triplets monozygotic.
	mm^2.	per mille.	per mille.	per mille.	per cent.
Stature	541·7	914·6	454·2	100·3	56·5
Stem length	227·4				
Span	651·0				
Cubit	55·38				
Head length	9·789	647·8	321·8	157·9	66·5
Head breadth	6·729	557·0	348·7	113·5	47·0

The mean percentage monozygotic is about 57 by this method. This estimate agrees well with one based on the sex proportions of the group of triplets concerned, for of the 44 cases, 10 belong to sets of 3 boys, 6 to sets of 2 boys and 1 girl, 15 to sets of 1 boy and 2 girls, and 13 to sets of 3 girls. The number of cases in which all the children are of like sex is thus 23, against 21 of mixed sex. The proportion of monozygotic like-sex pairs may be estimated as $3 \times 23 - 21 = 48$, against $2 \times 21 = 42$ dizygotic; a method of estimation which yields 53·3 per cent. monozygotic. Such a close agreement doubtless owes something to chance, while, on the other hand, it undoubtedly strengthens both estimates.

To obtain correlations between triplets of like-sex, comparable with those obtained for triplets of unlike sex, the individual variance for members of such pairs must be compared with the general variance of the population from which they were drawn. It was no part of the design of this research to obtain final figures for this variance, which, as was pointed out above, would be better based upon a comprehensive anthropometric survey; the data do, however, supply a tolerable basis for estimation, in the variance found among all children of like-sex who were measured. There were of these 49 boys and 68 girls. Since the girls also preponderate (28 pairs to 16) in the like-sex pairs, the variance obtained from these two groups may be pooled as they stand, giving an estimated variance with precision appropriate to 115 degrees of freedom. This does not preclude large sampling errors, but the results can be little affected at most by any real difference in variability which may exist between the two sexes. The like-sex correlation is then found by subtracting from unity the ratio of the individual variance of a member of a like-sex pair to the individual variance of a member of the general population.

The correlations thus obtained between triplets of like sex agree closely with those obtained by the author for the like-sex twins of Lauterbach's data.

Table X.—Correlations between Pairs of Like-Sex.

Triplets.			Twins (Lauterbach).		
	r.	z.		r.	z.
Stature	0·720	0·908	Stature	0·735	0·940
Stem	0·498	0·547	Stem	0·575	0·655
Span	0·735	0·938	Weight	0·735	0·938
Cubit	0·803	1·107	Cephalic index	0·731	0·931
Head length	0·766	1·010			
Head breadth	0·764	1·006			
Mean	0·726	0·914	Mean	0·699	0·866

In both series stem length shows a surprising departure in the direction of low correlation. In Lauterbach's data the effect could merely be noted as an anomaly; in the triplet data, though not in the twin data, it is also the lowest correlation for children of unlike sex. It was noticed early in the measurements that the values obtained for stem length were very sensitive to the posture in which the child was placed. Care was therefore taken to eliminate as far as possible such variations; the children were seated on a firm horizontal surface, usually a kitchen table, with their knees projecting as little as possible beyond the edge. I have, however, still suspected that the clothing, and especially the habitual sitting posture, of the child might considerably affect the measurements. In view of the slight discrepancies between duplicate measurements taken at about half an hour's interval, which only account for a depression of the value of the correlation by 0·014, the conclusion that difficulties of measurement are alone responsible cannot be regarded as established, and it may be that this measurement, unlike others, admits, at least in the growing child, an appreciable amount of environmental modification. Such a view is not free from difficulty, in view of the fact that with pairs of unlike sex the triplet data show little, and Lauterbach's data no effect, and, in addition, the effect with twins of like sex in Lauterbach's data is much more pronounced with boys than with girls. The discrepancies obtained from Lauterbach's measurements and my own should in any case be sufficient to put investigators on their guard against basing important conclusions on this particular character, without a thorough scrutiny of the reliability of the measurement technique to be employed.

If stem length is set aside as of doubtful reliability, the mean correlation from the triplets, obtained as usual from the values of z ($=\tanh^{-1}r$), is 0·759, while that from Lauterbach's twins is 0·734. Neither with nor without stem length are the values significantly different. This agreement supplies a further confirmation of the estimates made above of the proportion of monozygotic pairs, for this proportion was estimated in Lauterbach's data to be about 59 per cent.

Further and concordant evidence of the proportion of pairs to be regarded as monozygotic may be obtained from the values of the correlations themselves. Even if it were assumed that monozygotic twins were identical in their physical measurements, then if the dizygotic like-sex pairs were no more highly correlated than pairs of unlike-sex, it would require 47 per cent. monozygotic to raise the correlation from 0·545 to 0·759. This percentage may therefore be regarded as a minimum. If, on the contrary, we take the proportion as estimated by comparison with Dahlberg's data for the relative variance, and from the sex distribution (namely, 57 per cent.), then the correlation between monozygotic twins should be 0·920 in order to reproduce our correlations. A similar estimate made from Lauterbach's data gave values between 0·92 and 0·93 for all three characters other than stem length. In the values from the triplet data also we have omitted stem length from the average, and have also, on the other hand, made no allowance for the known errors of measurement which will have depressed all the other correlations by about 0·008.

Since an intra-class correlation measures the fraction of the variance ascribable to differences between the classes compared, a correlation of 0·93 between monozygotic twins may be interpreted as meaning that 93 per cent. of the human variance in physical measurements is to be ascribed to hereditary factors, leaving 7 per cent. for other causes of variation. Exactly what these other causes of variation are, it is extremely difficult to indicate. Gross factors, such as the supply of nutrients, facilities for exercise, and all other conditions open to inspection and regulation, may have very little influence; these would certainly cause smaller variations between twins than between unrelated children. Other causes, such as the onset, and age of onset, of infectious diseases, depend from less obvious environmental differences, and their effects will in consequence have a more accidental character. If, as seems probable from the mutual interaction of the parts in development, very minute initial variations of growth of particular organs may in some cases lead to appreciable ultimate difference in the measurements, these will be as great for twins as for unrelated children. Indeed it may be suspected that they will be greater, for the great differences in weight at birth show that the pre-natal condition of children born together must in some respects be strikingly different.

An exact estimate of the average correlation in metrical characters between identical twins should evidently combine a critical diagnosis based upon Dahlberg's methods, carried out independently by two different judges, without knowledge of the measurements; an equally necessary factor in such an estimation, and one which is unfortunately wanting from Dahlberg's data, is the value of the variance at a given age of the general population sampled. Experts differ profoundly as to the correct diagnosis of monozygotic and dizygotic twins (11, 12); nevertheless, the concordant results obtained by comparing the triplet data with Dahlberg's averages have convinced the writer that his method of diagnosis, which was published during the progress of the triplet enquiry, has gone far to solve the problem of discriminating the two types of twins. Such discrimination can, however, never be absolute, if only because genetic identity itself is not impossible for dizygotic twins.

The question whether all three triplet children can ever be regarded as monozygotic should evidently be answered confidently only on a larger basis of observation than the six cases for which measurements were obtained. A rough criterion based on the measurements may be made by excluding all cases which differed in any measurement by more than a fixed maximum difference. Taking arbitrary standards proportional to the standard differences between like-sex pairs, namely, for stature 40 mm., for stem length 26 mm., for span 44 mm., for cubit 13 mm., for head length 5·4 mm., and for head breadth 4·5 mm., only 22 pairs remain sufficiently alike in all measurements. These will doubtless be predominantly monozygotic pairs, but the criterion is far from decisive, and some overlap will doubtless have occurred. In no case do all three triplets fulfil the conditions. In three cases, numbers 1, 59 and 61, one child is considerably larger than the other two. In cases number 1 and 61 the two smaller children are alike in their measurements, in case number 59 they differ by 8 mm. in head breadth; this case is probably trizygotic. In the three other cases monozygotism cannot be confidently denied. In case 52, (*c*) is in most respects the smallest child, but only differs distinctly from her sisters in being shorter (by 5·6 and 7·2 mm.) in the head. In case 2 all three girls agree closely save that (*c*) is narrower (by 5 and 7 mm.) in the head. In case 40, (*a*) is both shorter in sitting height and narrower in the head than her two sisters. Since the standard difference in head length between monozygotic pairs, as judged either from Dahlberg's data or from the triplet measurements, cannot differ much from 3·1 mm., the case for regarding case 52 as monozygotic is particularly strong.

6. *Inheritance of Tendencies favouring Multiple Births.*

Much confused controversy has turned upon the inheritance of tendencies favouring twin births, and especially upon the influence of the father upon the production of twins. The most self-consistent view seems to be that developed by Weinberg, namely, that there is no paternal influence, and that the production of monozygotic twins is not inherited. This view is not only self-consistent but is supported by a considerable quantity of genealogical statistics; it appears indeed, without critical scrutiny, to be the only view consistent with the figures which Weinberg presents. Unfortunately, it has not been borne out by any other substantial body of data. The situation involves a definite conflict of evidence, and it is undesirable that this prime fact should be obscured, although it must necessarily be complicated by the conflict of inferences drawn from the evidence. The best hope of progress evidently lies in obtaining data from entirely fresh sources, taking every care to eliminate those causes of error which seem most likely to have produced the existing discrepancies.

A further point of importance is that conclusions should be based whenever possible upon comparisons made within a homogeneous body of data, rather than with data obtained by a different method, for apart from the potent influence of unconscious selection, differences in the completeness with which cases of twinning involving one or more still-births are recorded will much affect the frequency of twinning observed. That this precaution is not unnecessary may be seen from the great differences in absolute frequencies obtained by different observers in districts in which the official statistics show no comparable discrepancies.

The triplet data were entirely unselected save for the fact of the occurrence of triplets. Of the 148 families asked for information concerning related births, 98 provided complete and satisfactory information. It will be preferable to use only this unquestionable portion of the data; only one case of those omitted need be mentioned separately, namely, a family of 11 births, of which 2 were of triplets and 5 of twins, yielding in all 20 children. The inclusion of the case would evidently have much raised the observed percentage of twins in the families containing triplets, and possibly also in related families. Its omission, however, cannot invalidate the conclusions to be drawn from unquestionably correct information, for it is omitted not because the information was exceptional in character but because it was confused and very incomplete. A number of such cases of exceptional fecundity have been reported from time to time, and may represent a phenomenon distinct from the normal, though rare and scattered, occurrence of multiple births.

Full data for related multiple births are shown in Tables XI, XII and XIII. Among the families in which the triplets occurred, 493 other births are recorded, of which 14 were of twins and 4 of triplets. Counting triplets as equivalent to the production of twins twice (for, in addition to the production of twins, a second fission or additional ovulation must have occurred), the percentage of twinning in these families is 4·5 per cent. The ratio of triplets to twins is high, but this may be no more than a coincidence. It is to be presumed that the proportion of twins in Great Britain and Ireland is not greatly different from 1 per cent., and the other data of this enquiry show that it cannot be much more. Consequently a rate of 4·5 per cent. represents a very considerable concentration of multiple births, and shows that whether the tendency is inherited or not, certain parents are definitely characterised by higher twinning rates than are others.

Table XI.—Other Children of Parents.

Type of triplets.	Families.	Single births.		Twins.			Triplets.			
		Boys.	Girls.	2 boys.	1 boy, 1 girl.	2 girls.	3 boys.	2 boys, 1 girl.	1 boy, 2 girls.	3 girls.
3 boys	13	33	29	—	4	1	—	—	—	—
2 boys, 1 girl	27	79	73	—	1	—	—	—	1	1
1 boy, 2 girls	39	82	81	2	3	1	—	1	—	—
3 girls	21	45	53	1	1	—	—	1	—	—
Total	100	239	236	3	9	2	—	2	1	1
		475		14			4			

Table XII.—Children of Mother's Parents.

Type of triplets.	Families.	Single births.		Twins.			Triplets.
		Boys.	Girls.	2 boys.	1 boy, 1 girl.	2 girls.	3 boys.
3 boys	13	45	48	1	3	—	—
2 boys, 1 girl	27	80	102	—	1	2	1
1 boy, 2 girls	39	125	161	3	4	1	—
3 girls	21	76	89	—	1	2	—
Total	100	326	400	4	9	5	1
		726			18		

Table XIII.—Children of Father's Parents.

Types of triplets.	Families.	Single births.		Twins.			Triplets.
		Boys.	Girls.	2 boys.	1 boy, 1 girl.	2 girls.	
3 boys	13	62	41	1	—	—	—
2 boys, 1 girl	27	114	85	3	—	2	—
1 boy, 2 girls	39	151	120	3	1	2	—
3 girls	21	85	59	1	1	1	—
Total	100	412	305	8	2	5	0
		717			15		

Evidence of heredity must, of course, be drawn from the corresponding data for related families. Of 745 births to parents of the mother, 18 were of twins and 1 was of triplets; calculated as before, this gives 2·7 per cent. twins in these families. Similarly of 732 births to parents of the father, 15 were of twins; this is 2·0 per cent. The statistical significance of both results may be gauged by the fact that in a Poisson series with mean 7·3 a value as high as 15 will only occur eight times in a thousand trials.

The data for the families of the parents of triplets thus give definite support to the view that the production of multiple births is influenced to an important extent by the hereditary qualities of the father. Of the similar evidence from the relatives of the parents of twins, perhaps the most comparable is that given by Danforth (6) from St. Louis. It is stated that "the investigator had no knowledge of any of these families until in each case the birth of twins was reported to the bureau of vital statistics." Fifty families were investigated; of 181 births in the same families 10 were of twins (5·5 per cent.), of 328 births to parents of the mothers 10 were of twins (3·0 per cent.), while of 297 births to parents of the father 8 were of twins (2·7 per cent.). The twin data are smaller in volume, being based upon 50 families against 98 available for the triplet enquiry; the twinning percentages are distinctly higher in each case, although the families were selected merely by a single occurrence of twins, instead of by the occurrence of triplets; but the main conclusion that the selected families show the highest twin frequency, and that their relations on both sides show the same effect to a diluted extent, appears clearly from both enquiries.

Davenport (5) gives figures for a somewhat more highly selected group of families who have had twins twice. Of 355 births to the parents of twin-repeating mothers 16, or 4·5 per cent., were of twins; of 289 births to the parents of twin-repeating fathers 12, or 4·2 per cent., were of twins. The discrepancy in the actual percentages is here more striking than in those of Danforth, and we have in this case no assurance that cases of special interest do not find their way, more readily than cases showing no heredity, into the archives of the Eugenics Record Office. That a somewhat severe concentration of interesting cases may have occurred in this material is suggested by the percentage of twin births obtained for the brothers and sisters of repeating fathers and mothers. These are father's sisters' children, 8·2 per cent.; mother's sisters' children, 5·5 per cent.; father's brothers' children, 6·5 per cent.; mother's brothers' children, 4·5 per cent.

These high percentages, while apparently supporting, in reality introduce a difficulty to the theory developed by Davenport, for he infers from them equal paternal and maternal influence. Now if we select a person specially characterised, such as a twin-repeating father, or a twin-repeating mother, we may infer on this theory that both the parents of this person will (in less degree) be similarly characterised, and the children of the marriage between two such parents will therefore be twins with nearly double the frequency which would have been realised if only one of the parents had been so characterised; although, therefore, a brother or sister is genetically as close a relative as a father or mother, the theory is not compatible with the view that the sister or brother, married to some quite unselected person, will have twins as frequently as will the father and mother married to each other.

If 6 per cent. twins is correct for the brothers and sisters of the chosen parents, we might reasonably expect 11 per cent. twins from their fathers and mothers; on the other hand, if 4·4 per cent. is correct for the fathers and mothers, we might reasonably estimate the percentage for the brothers and sisters at 2·7 per cent. If heredity is at work it should, in fact, be made manifest not only by the contrast between the selected families and the general population, but equally by the contrast between different degrees of affinity to the *præpositus*, or selected centre, of each pedigree. The latter type of contrast is indeed the more important, since, as in England, the general population may be almost unknown, and, in other cases, as the divergences between different enquiries indicate, discrepancies of unknown origin have certainly been introduced between the official and the genealogical data ; consequently, in view of these, the comparison of different groups of relatives in the same material provides undoubtedly the best controlled tests available of the hypotheses to be considered.

One probable reason why this contrast, to be expected between different groups of relatives, has not been realised, or at least utilised, lies in the fact that the groups of relatives appropriate for comparison will be different, according to whether paternal influence is admitted or not. If paternal influence were absent, not only would the relatives of the father and the mother's brothers show no excess of twins, but the sisters and daughters of the mother should show no less effect than the mother's mother. This contrast between the twin-frequency of the mother's sisters on the one hand with the mother's brothers, the father's sisters and the father's brothers on the other, seems to provide a well-controlled method of determining if any important group of the causes of multiple births are limited, as Weinberg suggests, to action through the mother. Equally, however, the contrast between the twin-frequency exhibited by the parents and that of the brothers and sisters must be manifest if to any important extent twinning is inherited through both parents alike. Neither contrast appears in Davenport's figures in spite of the high percentages of twinning observed, a circumstance which makes it impossible to use these results with confidence as a means of discriminating between the two opposed views.

Much lower percentages of twins are found among the relatives of twin-bearing parents by Weinberg and Dahlberg. From Wurtemburg Weinberg finds 101 fathers of twins from families with 901 children in all, among whom 6 pairs are twins, thus showing no indication of paternal influence. More extensive data for the relatives of mothers are given for Stuttgart (3, p. 107).

Table XIV.—Related Births to Mothers of Twins.

	Relatives of mothers of twins of like sex.			Relatives of mothers of twins of unlike sex.		
	Mother.	Sister.	Daughter.	Mother.	Sister.	Daughter.
Multiple births	61	55	44	45	24	27
Single births	3909	2524	3386	1803	998	1437
Total	3970	2579	3430	1848	1022	1464
Percentage	1·53	2·13	1·28	2·50	2·35	1·84

The percentage of twinning is seen to be much lower than in either of the American groups of data discussed, that for the mothers being more comparable with the values obtained from the triplet data. For each degree of relationship the relations of twins of unlike sex have the higher percentage, and Weinberg concludes that only the tendency to dizygotism is inherited, that for diembryony showing no hereditary tendency. It should be noted that in Weinberg's material this difference is only statistically significant in the case of the mothers, though the daughters, and even to a very slight extent the sisters, show a tendency in the same direction. If the above figures are examined critically, it appears that the three classes of relatives do not give, within the limits of reasonable sampling errors, the same percentage of twins. This effect seems to be wholly due to the larger material on the relations of twins of like sex. In these the sisters show much the higher percentage. It is possible to give a plausible, though tentative, explanation of these discrepancies.

Dahlberg (3, p. 113) has criticised the material on the ground that incompleteness of registration may have considerably reduced the twin percentage. If this is so the sisters, which show the highest twin frequency, would seem to represent the generation in which the data are most complete, and it constitutes a serious *caveat* against Weinberg's conclusions—that all hereditary effects should be ascribed to dizygotism—that it is precisely in the sisters that no appreciable difference exists between the relations of twins of like and of unlike sex. Dahlberg has shown (3, p. 47) that the omission of still-births diminishes especially the percentage of monozygotic twins.

Upon the question of the inheritance of diembryony the triplet data appear to supply evidence of a decisive character. For of the 15 twin births of the parents of the father of the triplets only 2 are of opposite sex, whereas of the 18 twin births of parents of the mother 9 are of opposite sex. The whole of the twins, in excess of expectation for ordinary families, born to the parents of the fathers, are thus of like sex, and presumably therefore all monozygotic. It is not that every one of the 13 pairs found need be monozygotic, for two or three like-sex dizygotic pairs might well occur in 700 births taken at random. That 13 such pairs should occur is beyond reasonable probability, and since there is no evidence of any excess of dizygotic pairs, the plain inference from the data is that the paternal influence upon twinning is confined to the production of diembryony. Since in such cases fission occurs after the union with the egg of the paternal gamete, it is only natural that for this process developmental tendencies inherited from the father should be of equal importance with those from the mother. Whether the tendency to diembryony is also inherited through the mother, as seems *a priori* probable, could only be decided upon larger data, for the presence of an excess of dizygotic twins among the relations of the mother must much increase the sampling errors of any estimate of diembryony.

It is to be noted that all the groups of relationship data cited agree in giving a slightly higher twin percentage to the relations of the mother, though in no case, save that of Weinberg, is this excess statistically significant. It does, however, accord with the view that, while paternal influence only affects the one type of twinning, maternal influence may act upon both. If, further, it is assumed that the maternal influence in diembryony is equally potent with that of the father, the smallness of the differences usually found between the relations of the two parents makes it probable that heredity is less influential in inducing dizygotism than it is in inducing diembryony.

This view is the direct contrary of that held by Weinberg; on the other hand, it tallies with Davenport's finding even higher twin-frequencies among the relatives of monozygotic twins than among those of twins in general. It may, in addition, be supported by facts other than the twin-frequency of relatives. It is well known that the percentage of twins increases greatly with the age of the mother. Knibbs (9, p. 309) shows a threefold increase between 20 and 38. Comparisons of the sex distribution at each age, of which the most thorough is that of Dahlberg (3, pp. 21–28), shows that this increase is certainly largely and probably wholly due to a change in the frequency of dizygotic pairs. Evidently, then, dizygotism is much influenced by an environmental factor, age of mother, upon which hereditary influences can have but little effect. Diembryony, on the other hand, is apparently not influenced at all by this factor. In consequence, it is possible to hold that the probability of monozygotic twins depends wholly upon the hereditary nature of the parents, while in the case of dizygotism any evidence for heredity is bound to be much obscured by the mere effect of variations in the age at childbirth.

If a strongly heritable maternal tendency towards dizygotic twinning existed, it would appear in contrasting the twin-frequency of mother's sisters with those of mother's brothers, father's brothers and father's sisters. No such effect appears in Davenport's data, and the comparison cannot be made in Weinberg's. The triplet data are as follows :—

Table XV.—Frequency of Multiple Births born to Brothers and Sisters of the Parents of Triplets.

	Mother's sisters.	Mother's brothers.	Father's sisters.	Father's brothers.
Single	504	501	626	532
Twin	5	6	7	7
Triplet	1	—	1	—
Total	510	507	634	539
Percentage	1·4	1·2	1·4	1·3

No significant differences exist between the four classes, and it may be doubted if any of them differ significantly from the twin percentage of the general population. For this reason the sex distribution of the related twins is of no special interest. The actual types are, however, given below :—

Table XVI.—Sex Distribution of Related Multiple Births.

	2 boys.	1 boy, 1 girl.	2 girls.	3 boys.	2 boys, 1 girl.
Mother's sisters	—	2	3	—	1
Mother's brothers	2	1	3	—	—
Father's sisters	1	3	3	1	—
Father's brothers	—	4	3	—	—
Total	3	10	12	1	1

Among the relatives of triplets the frequency of triplets compared to that of twins is somewhat high. Among children of the same parents we have 4 triplets to 14 twins, among the children of their parents, 1 triplet set to 33 pairs of twins, and among the children of their brothers and sisters 2 sets of triplets to 25 pairs of twins. The numbers are too small to give more than an indication, but they suggest that in addition to the factors favouring multiple births in general, there may be others which favour the particular sequence of events, such as double ovulation followed by fission of one zygote, which gives rise to triplets.

It will be noticed that a perfectly clear contrast exists between the frequency of twins born to mothers (2·8 per cent.) and that born to sisters (1·4 per cent.) of mothers of triplets, in accordance with the other clear evidence which these data provide of paternal influence. There is, however, one factor which should be mentioned, not as probably producing the whole effect, but as possibly contributing to it. The strong age association of dizygotic twinning renders the frequency of this phenomenon open to interference by artificial contraceptive methods. If these are employed after the production of a few children, a proportion of the married women concerned will only be "exposed to risk" during the earlier parts of the reproductive period. Such a tendency would certainly reduce the proportion of dizygotic, though probably not of monozygotic twins, and it is only in dizygotic twins that any excess is to be looked for of the mother's sisters against the mother's brothers, and the father's brothers and sisters. If, as there is ample reason to believe, contraceptive practices were more widely employed in the generation represented by mother's sisters than in the previous generation of mothers' mothers, we might reasonably put down some part of the contrast to this cause. It should perhaps be mentioned in this connection that the frequency of triplets, or at least of applications for the King's Bounty, has fallen off in the last 25 years more rapidly than would be expected from the decrease in the annual number of single births registered.

7. *Summary.*

Measurements taken at a fixed age of 115 surviving triplet children show no distinct difference in stature from children by single birth. The precision of the comparison is, however, limited by our scanty knowledge of the average bodily growth in the general population of Great Britain and Ireland.

The average degree of resemblance between triplet children of opposite sex does not differ appreciably from that of twins of opposite sex, or of fully grown brothers and sisters by different births.

The differences between triplets of like sex are intermediate in magnitude between those found by Dahlberg in groups of like-sex twins classified as monozygotic and dizygotic respectively. The comparison indicates that about 54 per cent. of the like-sex triplets pairs measured were monozygotic. The correlation between triplet children of like sex, the estimate of which could be improved by a better knowledge of the general population, agrees well with that found for like-sex twins in Lauterbach's measurements, and indicates a correlation between monozygotic pairs of about 0·92.

The material is not sufficient to establish definitely that in any set all three triplets were monozygotic, though this is a probable explanation of the resemblance in at least one set. The discrepancies between the data provided by different investigators upon the inheritance of tendencies favouring multiple births have prevented any substantial agreement on this subject. An examination of the material suggests that these discrepancies are due (*a*) to the accumulation of interesting cases showing apparent heredity in pedigree collections; (*b*) inequality in the completeness with which still-births are recorded in the different sources of material compared.

To avoid these discrepancies it is important to spare no pains to ensure that a strictly valid statistical technique is employed in the collection of data, a consideration which is of equal importance in the statistical study of rare occurrences of all kinds; and to utilise, as the best controlled evidence of inheritance, the contrast in twin-frequency between different groups of relatives in the same collection. When allowance is made for these two sources of discrepancy, existing data upon twins may be brought into harmony with the results of the triplet enquiry.

The triplet pedigrees show a significant excess of multiple births in the families of both the father and the mother of the triplets. In the case of the father the excess consists wholly of like-sex twins. In other material the sex distribution of the related twins seems not to have been recorded, in spite of evident importance for the classification of heritable tendencies affecting dizygotism and diembryony respectively. The triplet data indicate that the paternal influence is only exerted in the production of diembryony. It is probable *a priori* that the heritable constitution of the mother exerts an equal influence in this respect, but more extensive data would be required to demonstrate this.

Since, in these and other data, the maternal inheritance is not much stronger than the paternal, it is probable that dizygotism is less strongly inherited than is diembryony. This view is supported by the well-established increase in multiple births with increasing age of mother, which effect appears to be confined to dizygotism.

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The Differential Effect of the Ions of Three-Salt Solutions on the Growth of Potato Plants in Sand Culture.

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Introduction.

A great deal of work has been carried out, mainly by American plant physiologists, on the effect of mixtures of three or more salts in varying proportions on the growth of plants in culture solution. In Tottingham's original work (1) a mixture of four salts was employed, consisting of seven ions; later the system was reduced by Shive to six ions. The plant employed in both cases was a variety of barley.

In 1924 E. S. Johnston (2) published a paper on "Growth of Potato Plants in Sand Cultures" treated with the "six types" of nutrient solutions. In this work the six ions employed by Shive (3) were used, but they were utilised as combinations in varying proportions of the six possible molecular groupings, giving six types of solution. As each series was in duplicate, a large body of data is available for a further analysis of the differential effects of the individual ions. In common with previous workers, Johnston was content to select, merely by inspection, the particular culture solutions which appeared to give the greatest yields.

An examination of the published data of all these experimenters discloses the fact that agreement is not always good between dry weights obtained from identical solutions in two parallel series of cultures, and, moreover, the composition of the solutions giving highest yield is not always the same in two parallel series.

In experiments of this kind it is evidently desirable to improve on this method of visual inspection in assessing the relative merits of solutions of different compositions. Furthermore, the full information embodied in the data has not been utilised; for instance, out of 123 cultures Johnston has utilised only 42 in attempting to arrive at the relative effects on growth of the ions concerned. Shive and Tottingham both used three sets of culture solutions of known total osmotic pressure, and claimed to show that the composition of the best solution varied with the concentration. An examination of the contours of yield in the graphical representation of their results leads one to suspect that chance errors played a large part, and as no estimate of variability was made, it is difficult to accept their claims.

Scope of the Present Paper.

In view of the importance of estimating the effect on yield of plants of various single nutrient ions, when these are presented in mixtures of salts, it has seemed advisable to attempt an analysis of existing data to this end. For this purpose the data published by E. S. Johnston have been utilised, since they are remarkably complete and each experiment is duplicated. Furthermore, they have evidently been carried out with great care and with a sound experimental technique.

The method utilised in this paper, as applied to Johnston's paper, has made use of all the experimental values provided. By using the method of least-squares, the variability of the material has been taken into account, and the values finally arrived at are those giving the best possible agreement with the experimental data on the basis of the assumptions made.

Criticism of Experimental Procedure employed by Johnston.

A full description of the procedure employed by Johnston will be found in his original paper, but it will be necessary to give a brief résumé of the method here. The six ions investigated were K', H_2PO_4', $Ca^{\cdot}$, NO_3', $Mg^{\cdot}$, SO_4', which were combined together to give the following six types of solutions :—

Table I.

I.	II.	III.	IV.	V.	VI.
$Ca(NO_3)_2$ KH_2PO_4 $MgSO_4$	$Ca(NO_3)_2$ K_2SO_4 $Mg(H_2PO_4)_2$	$Ca(H_2PO_4)_2$ KNO_3 $MgSO_4$	$Ca(H_2PO_4)_2$ K_2SO_4 $Mg(NO_3)_2$	$CaSO_4$ KNO_3 $Mg(H_2PO_4)_2$	$CaSO_4$ HK_2PO_4 $Mg(NO_3)_2$

Salts used in the six types of solutions employed by Johnston, each type consisting of a different molecular combination of the same six ions.

Quoting from Johnston's paper, " Each of the types one to six was composed of 21 different combinations of three salts. In each type the partial volume molecular concentration of each salt in the 21 solutions differed by increments of one-eighth of the total volume molecular concentration. The total osmotic pressure of each solution, however, was calculated to be 1·00 atmosphere."

Table II.

Type I.	Molecular proportions.			Partial volume—Molecular conc.		
Culture No.	KH_2PO_4.	$Ca(NO_3)_2$.	$MgSO_4$.	KH_2PO_4.	$Ca(NO_3)_2$.	$MgSO_4$.
1	1	1	6	0·0027	0·0027	0·0161
2	1	2	5	0·0025	0·0049	0·0123
3	1	3	4	0·0024	0·0071	0·0094
4	1	4	3	0·0022	0·0089	0·0067
5	1	5	2	0·0022	0·0108	0·0043
6	1	6	1	0·0020	0·0122	0·0020
7	2	1	5	0·0053	0·0027	0·0132

Reproduction of a portion of Table I in Johnston's paper, showing in the first half the proportions in which the salts are present, and in the second half the relative number of gram-molecules of each salt present in the various solutions.

The above table is a reproduction of a portion of Table I in Johnston's paper. The term Volume Molecular Concentration denotes gram-molecules per litre, and hence the figures in the second half of the table indicate the relative number of gram-molecules of each salt present in the various solutions. It will be seen in the above table that the total number of molecular proportions in each solution is always eight, but as the valency of the salts varies, the number of ionic proportions varies from 17 to 22. In order to maintain an equal osmotic pressure, the partial volume molecular concentrations vary in the different solutions. On recalculating the figures it is seen that the assumption has been made that complete dissociation of the salts has taken place. Tottingham and Shive both stress the importance of the dissociation constant (osmotic factor) in calculating the composition of their culture solutions.

Johnston made no determinations of osmotic pressure, and it can hardly be doubted that the total osmotic pressure was not constant in the different solutions. The *p*H factor was also left out of account, so that, apart from differences in composition, other factors also played their part in determining yield. The experiments with the different types of solution were done at different seasons of the year and over varying periods of time, and even duplicates were not carried through simultaneously, so that some doubts as to the comparability of the data in the various sets may be entertained.

Methods of Analysis.

The fundamental assumption made in the treatment of the data employed in this paper is that each ion works independently on growth, and that the total effect of all the ions is the sum of the effects of all the different ions. It is necessary to state that the total effect of the ions present may not be equal to the *sum* of the individual effects, but may be proportional to their product, or that the combined effect may be an even more complicated function of the individual effects. R. A. Fisher (4) has shown that response of potatoes to different manurial treatments is better fitted by a product formula than by a sum formula; but the assumption of simple summation simplifies the problem considerably.

To reduce the experimental values of growth in all series to a comparable basis, the mean dry weight for each individual series was found, and the separate dry weights for the 21 solutions were represented as a percentage of the mean. This, of course, assumes that the relative values for dry weight within the different series were comparable, and depend only on the "type" of solution used. The actual average values for dry weight in the different series, and in duplicates of the same series, vary greatly, but since the experiments were carried out at different seasons under different climatic conditions, it is impossible to compare directly the yields given by the different types, and thus to discriminate between the types as to their suitability for growth. Under the circumstances the only method possible is to reduce all the series to a comparable basis and to eliminate the seasonal effects.*

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* It is unfortunate that the seasonal effects had to be eliminated, for the reason stated, since precise information on the interaction of the climatic complex with manurial effects is one of the valuable contributions plant physiology can make to agricultural science.

The molecular proportions shown in Johnston's tables were replaced by ionic proportions (relative ionic concentrations), which were then equated against relative yields. Thus the portion of Johnston's Table I (reproduced as Table II above) becomes :—

Table III.

$$\begin{aligned}
a + b + c + 2d + 6e + 6f &= 0{\cdot}850 \\
a + b + 2c + 4d + 5e + 5f &= 0{\cdot}821 \\
a + b + 3c + 6d + 4e + 4f &= 0{\cdot}762 \\
a + b + 4c + 8d + 3e + 3f &= 1{\cdot}173 \\
a + b + 5c + 10d + 2e + 2f &= 1{\cdot}202 \\
a + b + 6c + 12d + e + f &= 1{\cdot}085 \\
2a + 2b + c + 2d + 5e + 5f &= 0{\cdot}938
\end{aligned}$$

$a = K^{\cdot}$, $b = H_2PO_4'$, $c = Ca^{\cdot}$, $d = NO_3'$, $e = Mg^{\cdot}$, $f = SO_4'$.

Equations showing relative number of ions of each kind present in the solutions of type I (presented in Table II) equated against the yield for the solution relative to the mean yield of all solutions taken as unity.

The equations given in the table show the relative number of ions of each kind present in the solutions of type I already presented in Table II. Each of the six kinds of ions is represented by a letter as indicated in the table, and thus a, $2a$, etc., represent 1, 2, etc., ionic proportions of potassium, present in the solution. The left-hand side of the equations thus represents the total number of ionic proportions of each ion present, and hence the total ionic content of the solution. On the right-hand side are given the yields for each solution expressed as a proportion of the mean yield of all the solutions of all types employed. This mean yield is taken as unity, and the yield for each solution stated as a proportion of unity. Since the effects of the single ions on yield have been assumed to be additive, the yield for a solution may be looked upon as the sum of the contributions to yield of the ionic proportions a, b, etc., on the left-hand side. By solving the equations the numerical value of these contributions to yield were found. In the way indicated above 123 equations were obtained showing the ionic proportions equated against relative yields in all the solutions of all types. These equations were solved simultaneously by the method of least squares, and gave "most probable" values for the variables. Two series of values are given by Johnston for each type and these were dealt with separately; thus it was possible to compare the values for the variables derived from two independent series of experiments. The 12 values thus obtained are given below.

Table IV.

	Series I.	Series II.
$K^{\cdot} = a$	+0·0696	+0·0723
$H_2PO'_4 = b$	−0·0127	−0·0160
$Ca^{\cdot} = c$	+0·1201	+0·1310
$NO'_3 = d$	+0·0491	+0·0476
$Mg^{\cdot} = e$	+0·0934	+0·0865
$SO'_4 = f$	−0·0001	+0·0023

Average contribution to yield of a single ionic proportion of each of the six ions in a solution containing all the ions in given proportions, and giving a yield of unity.

The figures given in the above table indicate the average effects of the individual ions over the whole range of solutions used. It should be emphasised that in the calculation of these values every figure for dry weight has been used, although some are obviously abnormal. The very high agreement between the values calculated from the two independent series adds greatly to the assurance of their accuracy.

The striking result is seen that in general the kations predominate greatly in their effects over the anions, and run in the order Ca, Mg, K. Of the anions only nitrate has a positive effect. Sulphate is practically without effect and phosphate is definitely adverse. In no previous investigation on the effect of mixed salts on growth have the effects of individual ions been separated. Experimentally this cannot be done, since ions can only be introduced into the system in pairs. Only by a process of analysis such as this can an estimate of their individual effects be obtained. Of the ions present two of the metallic ions are bivalent as against only one of the anions, which leads to the result that on the average the relative ionic concentrations (number of ionic proportions) in which each ion is present is not the same, viz., $8K^{\cdot}$, $10H_2PO'_4$, $6Ca^{\cdot}$, $10NO'_3$, $6Mg^{\cdot}$, $6SO'_4$. Since the ionic proportions of any particular ion vary from equation to equation, and all proportions are associated both with high and low yields, the tendency will be for the effects of the ions in lesser concentration to be overestimated, which may perhaps partly account for the high effects attributed to calcium and magnesium.

Johnston, in discussing his results, remarks that "in general it may be stated that higher averages of the two series (types I and II) occur for cultures containing relatively large proportions of calcium nitrate while the lower averages obtain for cultures low in calcium nitrate . . . Further analysis is, of course, necessary to determine whether the calcium or the nitrate is the governing factor." The further analysis undertaken in the present paper, in so far as it is adequate, indicates that both ions are concerned in the determination of the high yield associated with calcium nitrate, and indeed for each ionic proportion calcium predominates over nitrate in its effects.

It has already been stated that the total number of ionic proportions varies in the individual solutions of a given "type," and also is different for the various types of solution used. The reason for this is clear, since the salts used are both monovalent and bivalent, while the molecular proportions have certain determined values and always total up to a given number. The osmotic pressure, *i.e.*, the sum of the ionic proportions is, however, theoretically maintained constant, which means that in a series of solutions where the molecular proportion of a monovalent salt, such as KH_2PO_4, is constant (such as the series of solutions in Table II) the number of gram molecules per litre of KH_2PO_4 present will have to vary as the molecular proportions of the bivalent salt $Ca(NO_3)_2$ increase. Hence it follows that the total molecular concentrations of the solutions vary, and the actual weight per litre of a particular salt will differ among sets in which the molecular proportion of this particular salt remains constant.

The time elapsing between renewals of the culture solution is not stated in Johnston's paper, and should this period have been prolonged, the rapidly absorbed ions such as NO'_3 may have been completely removed from the solution. Both Hoagland[*] (5) and Burd (6) have shown that this occurs both in the soil solution and in sand cultures. Under such circumstances two solutions having at the outset equal molecular proportions of KNO_3 and different molecular proportions of $Ca(H_2PO_4)_2$, in spite of the equality at first of the number of ions of NO_3, would after a time differ in the number of ions remaining. This difficulty is inherent in the method of experimentation unless solutions are continually renewed.

These considerations have a bearing on the method of analysis used in this study. The sets of equations such as those in Table III do, in fact, give correctly the relative ionic proportions of the different ions present, but the sum total of the ionic proportions varies. It is assumed at the outset that the relative yield (as expressed in the right-hand term of the equation) is made up of contributions from each of the ionic proportions present on the left-hand side. Since the total number of these ionic proportions varies, whereas in the experiment, at least theoretically, the total number of ions was maintained constant to obtain constant osmotic pressure, the magnitude of the contribution to yield by each ionic proportion will decrease as the total number of ionic proportions increases. If then equations were selected with a fixed number of ionic proportions of one variable (say a) but with varying *total* number of ionic proportions, the numerical values of a as obtained from different sets would tend to decrease with increasing total number of ionic proportions. No preliminary method of treating the figures of relative yield, such as dividing in each case by the total number of ionic proportions present, would overcome the difficulty, since such treatment, to be adequate, would necessitate a knowledge of the relative magnitude of the contributions to yield of each ion, which is in fact the information required from the equations.

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[*] Hoagland gives figures showing absorption of ions of barley plants 7 weeks old: during 8 days from a solution of O.P. 1·72 1/7 total NO_3 absorbed, from solution 0·07 osmotic pressure 100 per cent. disappears.

Before describing the empirical method of approximation used to overcome the difficulty mentioned above, it may be well to consider briefly the average values of the effects of each ion as given in Table IV. Substituting for these values in an equation representing the average composition of the solutions all over the range, the figure for relative yield should come out at the average value, namely, 1·00.

The average solution has the following composition $3{\cdot}610a + 4{\cdot}528b + 2{\cdot}593c + 4{\cdot}512d + 2{\cdot}707e + 2{\cdot}593f$. Substituting the values of the variables given in Table IV, gives values for relative yield of 0·979 for series 1, and 0·983 for series 2. These figures clearly indicate that the values given in the Table do in fact represent the average effects of the ions; they are to be interpreted in the following way. In a solution of osmotic pressure of 1 atmosphere containing an ionic concentration of $K^{\cdot}$ of 3·610 parts in 20·543, roughly 2 in 11, 7 per cent. approximately of the yield in dry weight is contributed by each ionic proportion of potassium. Similarly for the other ions with positive effects. For phosphate ion the interpretation of the figure would be: the presence of an ionic concentration of $H_2PO'_4$ of 4·528 parts in 20·543, roughly 2 in 9, would decrease yield by approximately 1·5 per cent. for each ionic proportion of phosphate.

Empirical Method to Determine Approximations to the Real Values of the Variables.

All the equations were divided into groups consisting of those in which the coefficient of a particular variable is 1, 2, 3, etc., respectively. In these equations all the other variables except the first are replaced by their numerical values calculated from the average values in Table IV, *i.e.*, $2b$ was replaced by

$2 \times (-0{\cdot}032)$, etc. Each of the original equations thus gave a value for a, $2a$, $3a$, etc., according to the grouping. From these individual values the mean values for a, $2a$, $3a$, etc., were obtained. The process was then repeated for b, but substituting the new values for multiples of a. This method was carried out consistently in turn for increasing values of each of the variables, replacing in each case the variables already determined by their new numerical values until an entirely new set of values of increasing multiples of each variable was obtained. This gave the first approximation to the real values of increasing multiples of each variable.

It was now possible to draw graphs showing the effects of increasing amounts of each variable alone. These curves were smoothed to obtain more uniform values. The whole process was now repeated to obtain the second approximation. These values are shown in Table V.

Table V.

K.	H_2PO_4.	Ca.	NO_3.	Mg.	SO_4.
$a = 0{\cdot}026$	$b = 0{\cdot}068$	$c = 0{\cdot}158$	$d = 0{\cdot}017$	$e = 0{\cdot}213$	$f = 0{\cdot}032$
$2a = 0{\cdot}190$	$2b = 0{\cdot}032$	$2c = 0{\cdot}292$	$2d = 0{\cdot}045$	$2e = 0{\cdot}290$	$2f = -0{\cdot}048$
$3a = 0{\cdot}382$	$3b = 0{\cdot}005$	$3c = 0{\cdot}347$	$3d = 0{\cdot}100$	$3e = 0{\cdot}365$	$3f = -0{\cdot}048$
$4a = 0{\cdot}440$	$4b = -0{\cdot}040$	$4c = 0{\cdot}480$	$4d = 0{\cdot}162$	$4e = 0{\cdot}430$	$4f = -0{\cdot}040$
$5a = 0{\cdot}430$	$5b = -0{\cdot}065$	$5c = 0{\cdot}554$	$5d = 0{\cdot}220$	$5e = 0{\cdot}467$	$5f = -0{\cdot}033$
$6a = 0{\cdot}409$	$6b = -0{\cdot}112$	$6c = 0{\cdot}683$	$6d = 0{\cdot}260$	$6e = 0{\cdot}500$	$6f = -0{\cdot}024$
$8a = 0{\cdot}362$	$8b = -0{\cdot}183$		$8d = 0{\cdot}278$		
$10a = 0{\cdot}333$	$10b = -0{\cdot}255$		$10d = 0{\cdot}217$		
$12a = 0{\cdot}316$	$12b = -0{\cdot}325$		$12b = 0{\cdot}110$		

Average contribution to yield of increasing number of ionic proportions of each ion in a solution consisting in all of 20·5 ionic proportions, the remaining five ions being present in all possible combinations.

By summation the effects on yield of the ions in molecular combination were obtained and are reproduced in Table VI. These figures are represented graphically in fig. 1.

The Table gives the average contribution to yield of increasing numbers of molecular proportions of each kind of molecule in a solution consisting in all cases of 8 molecular proportions, the remaining constituents of the solution being salts of the other two metals, in all possible combinations. The figures in Table VI are graphically presented in fig. 1. The abscissæ represent the number of gram-molecules of each salt present as a fraction of the total gram-molecular salt content of the solution. The ordinates give the proportional contribution to total yield (taken as unity) of the various salts respectively.

Table VI.

	$Ca(NO_3)_2$.	$CaSO_4$.	$Ca(H_2PO_4)_2$.
1	0·203	0·190	0·190
2	0·454	0·244	0·252
3	0·607	0·299	0·235
4	0·758	0·440	0·297
5	0·771	0·521	0·299
6	0·793	0·659	0·358
	KNO_3.	K_2SO_4.	KH_2PO_4.
1	0·043	0·222	0·094
2	0·235	0·392	0·222
3	0·482	0·361	0·377
4	0·602	0·322	0·400
5	0·650	0·300	0·365
6	0·669	0·292	0·297
8	0·640		0·179
10	0·550		0·078
12	0·426		−0·009
	$Mg(NO_3)_2$.	$MgSO_4$.	$Mg(H_2PO_4)_2$.
1	0·258	0·245	0·245
2	0·452	0·242	0·250
3	0·625	0·317	0·253
4	0·708	0·390	0·247
5	0·684	0·434	0·212
6	0·610	0·476	0·175

Average contribution to yield of increasing numbers of molecular proportions of each type of molecule in a solution consisting in all of eight molecular proportions, the remaining molecules of the other two salts being in all possible combinations.

The curves do not call for any special comment, their smoothness, however, is striking. The predominating effect of the nitrates comes out very clearly, and up to the highest concentrations of KNO_3 and $Ca(NO_3)_2$ used the yield increases, but with $Mg(NO_3)_2$ high concentration definitely decreased yield. The general similarity between curves for nitrate and the type of curve demanded by Mitscherlich's equation of yield, is obvious.

The curves for phosphates and sulphates are more varied in form and not easily interpreted. The positive effect of the kation is partially neutralised by the adverse effects of the anions $H_2PO'_4$ and SO'_4; and in the case of $Mg(H_2PO_4)_2$ the negative effect predominates giving a falling curve. The method of deriving these curves indicates the possibility that the Mitscherlich curve may be the resultant of two effects, namely, of anion and kation respectively, whose separate relations with yield may vary within wide limits.

By summing the values in Table VI in the proportions of the molecular concentrations in Johnston's tables, the yield for each solution in the six types

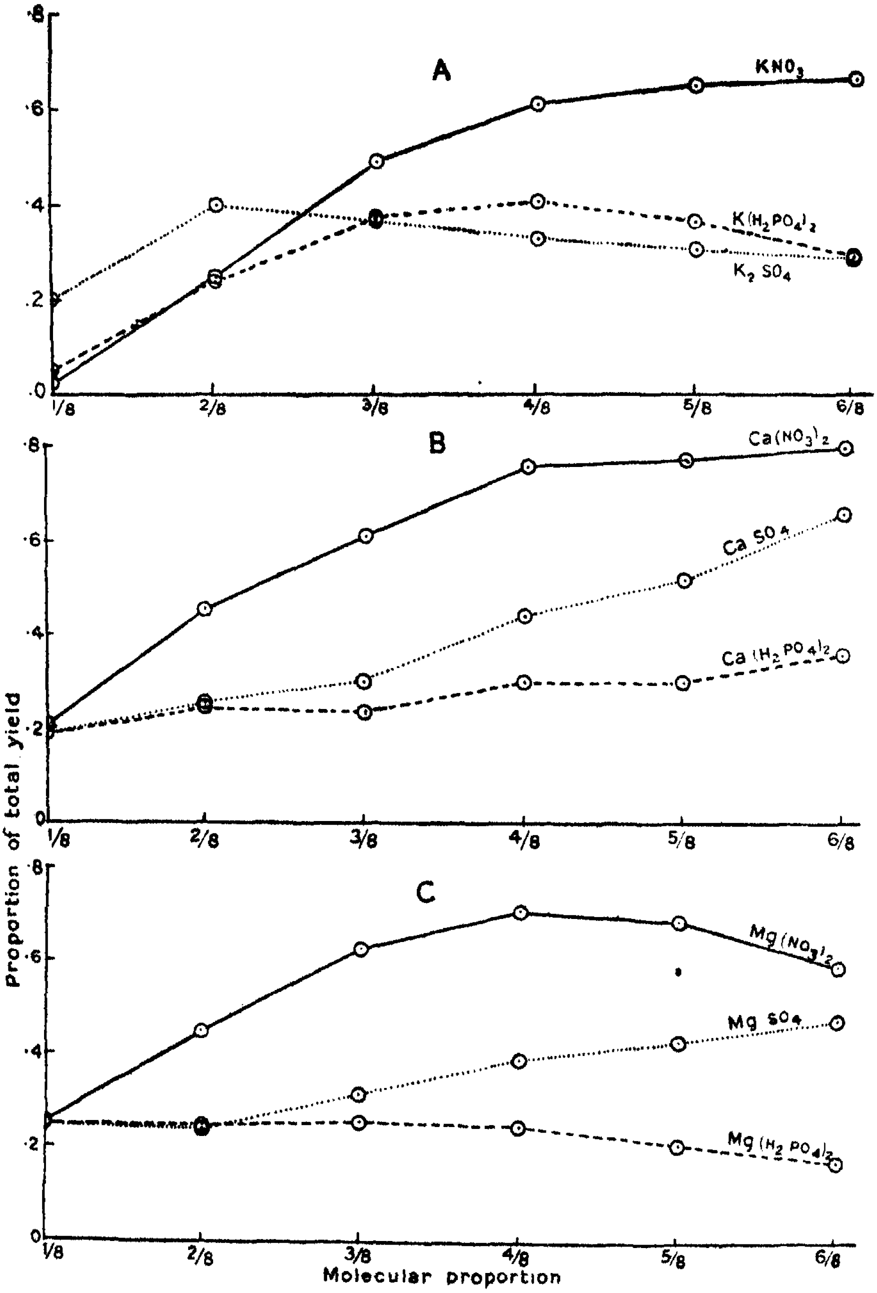

FIG. 1.—Graphical representation of figures in Table VI. Abscissæ represent number of gram-molecules of each salt present as a fraction of the total gram-molecular salt content of the solution. Ordinates represent proportional contribution to total yield (unity) of the various salts respectively.

may be predicted and has been calculated. A comparison of the calculated values with the observed values in Johnston's first series of experiments is found in Table VII.

Table VII.

Solution No.	Type I.		Type II.		Type III.		Type IV.		Type V.		Type VI.	
	Cal.	Obs.	Cal.	Obs.	Cal.	Obs.	Cal.	Obs.	Cal.	Obs.	Cal.	Obs.
1	0·77	0·85	0·60	0·42	0·71	0·71	1·02	0·92	0·41	0·47	0·89	1·04
2	0·98	0·82	0·89	1·04	0·73	0·85	1·16	0·90	0·50	0·55	1·02	1·04
3	1·09	0·76	1·08	1·32	0·67	0·53	1·17	1·00	0·59	0·82	1·10	1·07
4	1·17	1·17	1·23	1·00	0·66	0·85	1·14	1·18	0·74	0·59	1·16	1·04
5	1·11	1·20	1·24	1·38	0·58	0·53	0·97	0·98	0·81	0·51	1·07	1·45
6	1·13	1·09	1·26	1·14	0·65	0·67	0·84	0·88	(0·95)	—	(1·01)	—
7	0·86	0·94	0·81	0·62	0·86	1·13	1·27	1·33	0·64	0·78	1·10	0·89
8	1·07	1·32	1·09	1·04	0·88	1·34	1·35	1·98	0·73	0·78	1·17	0·95
9	1·15	1·47	1·25	1·04	0·79	1·02	1·25	1·18	0·79	0·94	1·15	0·95
10	1·22	1·23	1·40	1·14	0·77	0·74	1·14	0·98	0·93	1·02	1·11	1·07
11	1·24	0·97	1·41	1·70	0·78	0·71	0·95	0·76	1·00	0·90	(1·00)	—
12	1·97	0·70	0·81	0·66	1·06	1·24	1·26	0·92	0·92	0·90	1·28	1·18
13	1·15	1·03	1·07	0·87	1·05	1·13	1·24	0·88	0·98	1·25	1·25	1·04
14	1·23	0·97	1·22	1·04	0·96	0·99	1·05	1·06	1·03	1·13	1·13	1·17
15	1·38	1·38	1·36	1·45	1·02	0·99	0·92	0·71	1·17	1·13	1·08	1·01
16	0·92	0·82	0·78	0·76	1·11	1·41	1·14	0·96	1·05	0·98	1·22	0·86
17	1·10	1·06	1·03	0·97	1·10	1·17	1·03	1·08	1·10	0·94	1·10	1·18
18	1·25	1·14	1·17	0·97	1·08	1·10	0·82	0·84	1·15	1·29	0·96	0·80
19	0·81	0·70	0·75	0·76	1·08	1·63	0·94	1·02	1·09	1·17	1·01	1·01
20	1·06	0·76	1·00	0·97	1·15	1·34	0·81	0·76	1·14	1·17	0·87	0·68
21	0·75	0·65	0·74	0·69	1·10	0·92	0·74	0·71	1·10	2·66	0·75	0·68

Comparison of observed relative yield for each solution of the six types with the predicted yields obtained by summing the contributions to yield of the molecules present.

In Table VII the observed relative yield for each solution of the six types is compared with the predicted yield obtained by summing the contributions to yield of the molecules present, using figures given in Table VI, and molecular proportions as given in Johnston's Tables I to VI. The agreement between observed and calculated values all over the range is good, and by summing the squares of the differences between these, the variances for each type were found. They are as follows :—

Type.	Variance.
I	0·6816
II	0·5843
III	0·9231
IV	0·9114
V	2·8101 (0·3765)
VI	0·5799

Except for type V the variances do not differ much and indicate that agreement is equally good for all types of solution used. The large variance in type V is due to the abnormally high value of relative yield in solution 21, and if this figure is neglected the variance falls to 0·3765, which is comparable with the rest. The total variance is 6·4904.

When the two series of experimental values for relative yield are compared in the same way, the variances are as follows :—

Type.	Variance.
I	1·7947
II	1·3789
III	0·9256
IV	1·2227
V	2·7172 (0·4068)
VI	1·1962

In every case except type V, and also in this case when solution 21 is neglected for reasons stated above, the variances for differences in experimental values in the two series is greater than for an experimental set of values and the corresponding predicted values.

The total variance for differences between the two experimental series of values is 9·2630, while for differences between predicted values and the values in series I the total variance is 6·4904, which is very much less. Hence it follows that the calculated values agree with experimental value better than two experimental series agree among themselves. More cannot be expected of predicted values.

The total variance (Σd^2) between experimental values makes possible an estimate of their probable error. For a single dry-weight figure this works out as 18·5 per cent. The predicted values are presented graphically in fig. 2.

The method of plotting the results is briefly as follows. Each side of the triangles represents the base-line from which the quantity of a particular salt present in a solution is measured. It is a well known geometrical fact that the perpendiculars drawn from a point within a triangle on the three sides always have a constant sum-total in length. From this it follows that if the perpendicular distance from any point within the triangles represents the percentage of the salt, for which that side is the base-line of measurement, present in a solution, then each point within the triangles will represent a mixture of the three salts in certain relative proportions, in solutions which always have the same total number of molecules. The composition of the 21 solutions of each of the

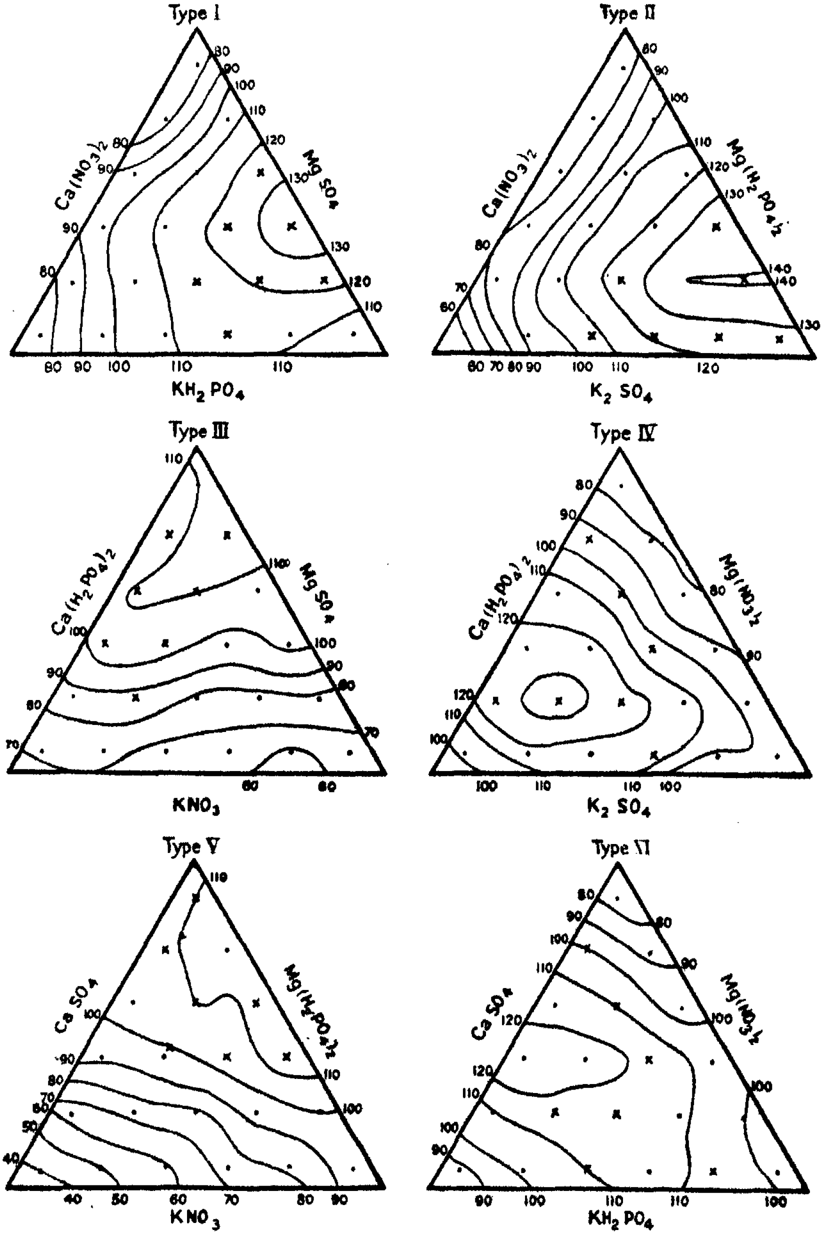

FIG. 2.—Contours of relative yield for solutions of three salts in all combinations represented by points within the triangles. The perpendicular distances from a point to the sides of a triangle are proportional to the percentages, in the solution, of the three salts inscribed on the three sides respectively.

six types is represented by the points and crosses within the triangles in the diagram.

Numbers representing the predicted relative yields were inserted at the points corresponding to the positions of the solutions within the triangles, and contours of relative yield were thus constructed, joining points of equal predicted yield.

The separate values for relative dry weight are not inserted in the diagrams but the contours of yield are numbered, and give a clear picture of the distribution of relative yields for the various types of solution. The crosses mark the positions of the seven best cultures in each set which Johnston used for estimating the effects of the ions. In general they occupy the positions round about the contours of highest yield. The scattered positions of the seven best cultures in types IV and VI were commented on by Johnston, but this is clearly related to the wide spacing of the contours in these two sets. Types I and VI differ only in the interchange of NO'_3 and SO'_4 ions between calcium and magnesium. The contours are almost mirror images of each other. This is equally true of types II and IV, but the interchange of SO'_4 and $H_2PO'_4$ in types III and V does not lead to a reversal of contours. In the latter case, the contours run more or less parallel to the base of the triangle.

In general the contour lines will tend to run parallel to that side of the triangle from which the proportion of the dominant ion is measured. Thus in the case of types III and V this tendency is well represented by the effect of the nitrate ion, although the effect of the Ca ion is seen for the contour of highest yield in type III, and in the general deflection of the contours towards the calcium side of the contours of type V. The high effect of the calcium ion is best seen in types I and II, where the contour lines run almost parallel to that side from which the proportion of calcium is measured. Here again the lines of highest yield show a deflection parallel to the potassium side, indicating the subsidiary effect of this ion.

In types I and II it so happens that both $Ca^{\cdot}$ and NO'_3 are associated on the same side of the triangle, and their effects on the run of the contours reinforce each other. The same tendency is seen with $Mg^{\cdot}$ and NO'_3 in types IV and VI. Johnston's conclusion that the best cultures always tend to occupy the positions as far as possible from the nitrate base-line is well brought out in all the diagrams. The reason for this is clearly that all the kations having high effects on yields, their individual effects will not greatly affect the run of the contours, which are thus almost completely determined by the inter-relations of the anions.

As sulphate has been shown to have practically no effect, the general distribution of the contour lines will be determined largely by the opposing effects of NO'_3 and $H_2PO'_4$. Thus the lines will always tend to run parallel with the nitrate base-line and meet the phosphate base-line at a high angle. This tendency is evident in every case irrespective of the distribution of the kations. A similar relation holds for the sulphate base-line, and is seen to occur.

From the distribution of the contour lines the predominating effect of nitrate is very obvious, and the correct conclusion as to the effect of nitrate ion was arrived at by Johnston; the strong effects of the kations could not be predicted by mere examination of the distribution of the best cultures within the triangles, and Johnston's conclusion of magnesium having an adverse effect is not substantiated.

It may be claimed that the results of the method of analysis outlined in this paper have thrown a considerable amount of light on the complex problem of the interaction of ions in solution. The application of this method to other experimental data of the same kind could not fail to yield important information on the nutritional requirements of other plants, and their specific relations to the ions investigated.

In conclusion the author wishes to record his thanks to Mr. F. Crowther, of this Department, for assistance in the labour of calculation.

Summary.

1. The paper describes a method of statistical analysis which enables an assessment to be made of the effect of single ions on the growth of plants in a culture solution consisting of a mixture of salts.

2. The method has been applied to a very complete set of data published by E. S. Johnston in a paper entitled "Growth of Potato Plants in Sand Cultures treated with six 'Types' of Nutrient Solution."

3. The experimental procedure employed by Johnston is considered and the shortcomings of all similar methods indicated.

4. It is found that the kations in the solution have for each relative ionic concentration (ionic proportion) a greater effect than any of the anions used.

5. The effects of the six ions used are presented numerically and graphically both for the ions singly and in molecular combinations.

6. The predicted values for yield in the solutions employed by Johnston are compared with his experimental results, with the result that the agreement between the two values proves to be closer than the agreement found between duplicate sets of experimental results.

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On the Production of Silk by Species of the Genus Hilara *Meig. (Diptera).*

By H. Eltringham, M.A., D.Sc., F.Z.S., with an Appendix on the Habits of the Species, by A. H. Hamm, A.L.S., F.E.S.

(Communicated by Professor E. B. Poulton, F.R.S.—Received Oct. 17, 1927.)

[Plate 22.]

In the *Entomologist's Monthly Magazine* for 1877, p. 126, there is a note by Baron C. R. Osten-Sacken describing certain species of the genus *Hilara* which, in flying, carried with them "bits of white tissue." These objects, on closer examination, proved to be flakes of "opaque, white, film-like substance, oval, about 2 mm. long, and so light that the faintest breath of air could lift them." He describes the appearance of the film as "not unlike the opaque white tissue spun by some spiders." The species of fly was identified as *Hilara alpina*, Loew, by Prof. Loew, who also informed Baron Osten-Sacken that Prof. Zeller had made a similar observation on August 14, 1873, near Bergün.

The foregoing account appears to include the first recorded observation on the habit of silk-spinning in the genus. In regard to the name *alpina*, the original identification of the film-making species is questioned by Becker (*Berlin Ent. Zeit.*, 1888, p. 7) and he gives to it the name of *Hilara sartor*, and appends a full description of the two sexes. He supposes that the secretion is produced from the abdominal glands. In 1889 (*Ent. Nachr.*, p. 220) Girschener described the webs made by *H. maura*, Fab., and *H. interstincta*, Fall., and pointed out that they sometimes contained the insect prey of Hilara. Later, Mik added the species *H. aëronatha* to the list of those which carried prey. Handlirsch (in *Verh. Zool.-Bot. Ges.*, Wien, 1889, p. 624) further described, with much detail of measurement, the webs or veils made by *H. sartor*. He gives the diameter of the threads as 0·001 to 0·0015 mm., and states that the silk is similar in chemical composition to that of caterpillars and spiders.

In *Wiener Ent. Zeit.*, vol. 12, p. 260, Mik published a paper on the biology of certain Diptera, the second part of which is devoted to the spinning of certain Hilaras. He discusses at length the previous work on the subject, and the many opinions that have been expressed as to the use, method of carrying, and origin of the silk, and concludes that it is produced from the mouth. In the *American Naturalist*, 1899, No. 394, p. 809, Aldrich and Turley give an account of a "Balloon-making Fly." The species was identified as *Empis poplitea*, Loew, "or a closely allied species." In this case the balloon is described as composed of sticky globules, which were immediately dissolved by alcohol. The balloons sometimes contained prey.

Evidently in this species we have something of a different nature from the silk produced by Hilara. In the silk-making species of Hilara, the substance hardens so rapidly in the air that it is never found to be sticky, and it consists of threads and not globules. Mr. A. H. Hamm has, however, found a species which carries small globules apparently of a nature somewhat different from the usual silk.*

It would be difficult, even in a paper of much greater length than this, to do adequate justice to the long series of painstaking observations which have been made by Mr. Hamm, of the Hope Department at Oxford, on the habits of the British species of Empidæ, including especially the genus *Hilara*, found near Oxford. The first published account of this work appeared in the *Oxford University Gazette* of 4th June, 1913, being in fact a part of Prof. Poulton's Report upon the Hope Department for 1912. The collections have been generously presented to this Department and are arranged in groups—"first and lowest, as food devoured by both sexes without relation to pairing, then as

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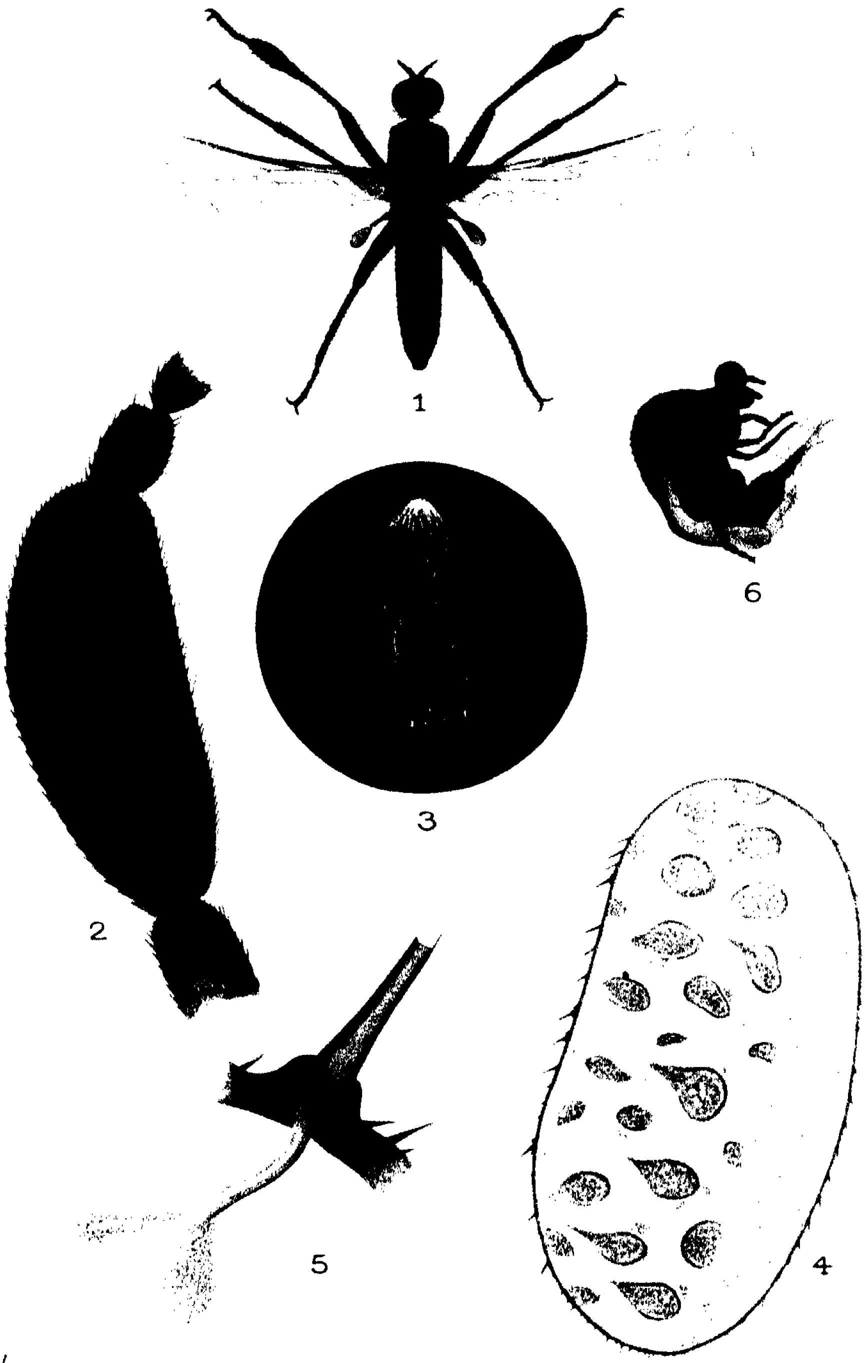
* The precise nature of these globules remains in doubt. The species found carrying them has been identified by Mr. J. E. Collin as *Hilara maura*. I have made preparations of the elaborate genitalia both of *maura* and of an individual which Mr. Hamm found carrying a globule, and could detect no difference between them. The globules are of a peculiar viscid substance. So permanent are its qualities that examples mounted on cards fifteen years ago are still as viscid as when first obtained. The substance is soluble in water, rather slowly soluble in xylol, and insoluble in pure alcohol, ether or chloroform. It has a sweet taste. I described these properties to my friend Mr. D. L. Chapman, F.R.S., the well-known chemist, and he is of the opinion that it must be of a sugary nature. Since *H. maura* will pick up any small object, it seems likely that the globules are obtained by it from some external source, but further investigation can only be made during some future season when the fly is obtainable.



H. Eltringham del.

Production of Silk by Flies of the genus Hilara.

a gift provided by the male and devoured by the female during pairing, finally as it were an ornament or plaything—no longer eaten by the female, but acting as a lure or stimulus. In the last stage the prey is often replaced by some vegetable fragment which is quite unsuitable as food. The climax of this line of evolution is reached in an elaborate cocoon spun by the male around the prey, and replacing the latter as an object of attraction " (see *Ent. Mo. Mag.*, vol. 49, 1913, p. 178, where the report is reprinted).

Amongst the species experimented upon by Mr. Hamm are the following—*H. maura*, Fab. This species makes quite considerable " balloons " of which one is illustrated in fig. 3, Plate 22. This particular example is about 4 mm. long and 2 mm. wide, and contains a small object, probably a particle of vegetable débris. Although there is some irregularity, a general direction of the main threads can usually be detected, running from pole to pole, and where two join towards one end of the cocoon they often fuse into a single thread. The form of these cocoons varies greatly, and almost any shape may be found, from an elongated balloon, pointed at both ends, to a mere tangled, more or less spherical mass.

I have shown in the text-figures six examples of these cocoons. Nos. 1 and 2 were made by *H. pilosa*, Zett., 3 to 6 by *H. maura*. Sometimes a fairly regularly constructed example spreads out spontaneously into quite a large ball of fluff

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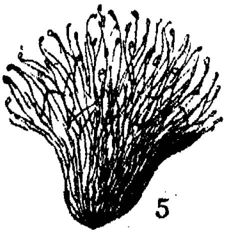
2

3

4



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6

after it has been mounted on a card. *H. maura* will accept any small object thrown on the water, though it often catches small insects, and even examples of its own species. When such prey is large, it seems to be merely rolled up and tied with a few strands of silk. Fig. 6, Plate 22, shows another Hilara which, after being disabled and thrown on water, was picked up and bound with silk by a male *maura*.

The manner in which *maura* and other species overcome the surface tension of the water in order to pick up a floating object is remarkable. The insect whirls rapidly round and round, and so lifts the prey in a little vortex of its own making.

H. quadrivittata, Mg., was not observed to make any balloon, but takes small insect prey and binds it with silk. It will not accept any extraneous object.

H. curtisi, Collin (= *cilipes*, Meig.), has the usual enlarged tarsal joint but has not been observed to make silk.

H. beckeri, Strobl., has the tarsal joint but slightly enlarged. It takes insects as prey and ties them with silk, but does not appear to make balloons.

H. pilosa, Zett., has been taken by Mr. O. W. Richards, of the Zoological Department, Oxford, and by Mr. Hamm, in very small numbers. It makes a silken structure of great beauty, which may be described as comet-shaped, having numerous threads trailing from a kind of central point or nucleus. The few examples obtained in the first instance (by Mr. Richards) had prey in the silk. Others taken later by Mr. Hamm had none.

H. thoracica, Mcq., is found to take either insect prey or small extraneous objects and bind them with silk.

H. litorea, Fall., taken by Mr. Hamm near the Oxford University Parks, has a well-developed enlargement of the tarsal joint, but the insect prey taken with it does not show any evidence of silk.

H. chorica, Fall., has a remarkable tarsal joint which is short and rounded. It takes insect prey, but there is no definite evidence of silk.

H. fuscipes, Fab. (= *carinthiaca*, Beck.) takes insect prey and ties it with silk.

All the above species, and others collected and observed by Mr. Hamm, can be seen in the Hope Department at Oxford. Only an inspection of the material can give an adequate idea of their collector's great industry and powers of observation. The dates extend over several years, and the mounts contain thousands of specimens, their cocoons and prey.

Though the production of silk by these flies has been known for so many years, the organs associated with it have remained undiscovered. The secretion has been variously supposed to emanate from the mouth and from abdominal glands. Some time ago Mr. Hamm suggested that I should endeavour to find the silk-glands, since such must evidently exist, and I did in fact make sections from material which he kindly obtained for the purpose. I must admit that I also was obsessed with the idea that the glands would be found in the thorax or abdomen of the insect. Indeed one would rather naturally expect to find some modification of the salivary glands. My researches did not then furnish any clue to the mystery, the short season of the imaginal fly was over, and pressure of other work caused the matter to be left in abeyance. Early this summer however (1927) Mr. Hamm again brought me material and suggested that the peculiarly enlarged basal joints of the fore-tarsi in the male might be worth examination. The first species to be taken was *H. maura*, and when this was over the work was continued with *H. quadrivittata*.

On teasing out one of the enlarged tarsal joints, which had been treated with picro-chlor-acetic acid, and examining the mass under a moderate power, there appeared numerous large cells or glands, somewhat pear-shaped, and each having a tube-like structure emanating from its thinner end, like the much enlarged stalk of the pear. Evidently there was some glandular structure in the tarsus.

Fresh examples were then examined. On breaking open the chitin there emerged drops of a clear fluid which, on exposure to the air, hardened with great rapidity. A needle inserted in one of the drops and drawn slowly away carried with it a thread which became dry and more or less rigid in a few seconds, the effect being similar to that obtained in drawing out a thread of molten glass. The substance in the tarsal joints strongly resembled a silk-like secretion.

Sections, both longitudinal and transverse, having been made, the glandular nature of the contents of the modified tarsal joint was no longer in doubt, and the tubular ducts from the cells, though very liable to fracture in section-cutting, evidently ended at the chitinous exterior, principally on the rather narrow edge of the tarsal joint. Examination of a piece of the chitin, flattened-out on a slide, disclosed the fact that close to the base of some of the spines in this area, there was, in the substance of the chitin, a minute pore, at first interpreted as a means of exit for the secretion. I now know, however, that this is incorrect, and the function of these pores remains for the time being undiscovered. The chitin is densely pigmented, and, being rather hard, it is extremely difficult to cut thin sections.

Having determined the part to which most of the gland ducts were directed, and prepared more sections, so oriented as to pass through this area, a careful examination showed that each gland-duct led to the base of a specialised spine.

Most insect spines, though hollow throughout the greater part of their length, are closed at the distal extremity. The poison-spines on the larvæ of Limacodid moths contain glands and are capable of injecting the poison into any wound they make, but this is accomplished by an ingenious arrangement by which the point of the spine becomes detached.* Ordinary examination of the spines on the tarsus of Hilara does not disclose any opening, and yet the gland-duct leads directly into the spine.

A perfectly fresh tarsus was placed on a slide under a medium power and pressure applied by laying over it another slide. Minute beads of the secretion could then be seen to exude from the points of the spines, and, by sliding the upper glass over the lower, these beads could be drawn out on the glass into threads which hardened rapidly in the air, and although thicker, resembled the silk spun by the insect. Sections in which these special spines occurred were examined under a high power, and it was then seen that the hard chitinous tube of the spine ended at a short distance below the extremity, and the point, which is comparatively blunt, seemed to consist of a very delicate membrane. There can be no doubt that the membrane opens at the end under the pressure of the secretion, and probably closes again by its own elasticity. Indeed such a provision would seem to be necessary, since the secretion hardens so rapidly on exposure to the air that an ordinary open tube would speedily be blocked by a film of the solidified substance at its extremity. In many sections ordinary spines can be seen, but in these the deeply pigmented chitin continues to the end, which is sharply pointed, and unprovided with the valve-like structure found on the spinneret-spines, as they may be called.

Reference to Plate 22 will now make these organs clear. Fig. 1 is a magnified view of the male of *Hilara maura*, in which the enlarged basal joints of the fore-tarsi can be distinctly seen. The wings are actually covered with minute hairs, but as these could not be resolved at the power used when making the drawing, I have not indicated them. The actual wing expanse of the specimen from which this was drawn is 11·5 mm. Fig. 2 shows the enlarged tarsal joint under a much higher magnification, and at the upper part of the right side the beads of the secretion can be seen exuding from the spines, as they do when pressed between two glass slips. Fig. 4 is a magnified view of the tarsal joint of *H. quadrivittata* as seen in longitudinal section. The drawing, which represents a reconstruction from several sections, as well as from teased and other preparations, shows the appearance when stained with eosin, methyl-blue orange-G, and light-green.

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* See my '*Butterfly Lore*,' Oxford, 1923, p. 50.

It will be seen that the secreting cells consist of an enveloping cytoplasm (stained pink), enclosing a mass of granular, globular, or vacuolated secretion (stained mauve), and where it happens to fall in the plane of the section, the duct of each cell can be traced to its junction with the chitinous envelope. The cells themselves show remarkably little structure, even when stained with iron-hæmatoxylin. The outer cytolasmic envelope appears slightly granular, and portions, occasionally darker, though ill-defined, seem to indicate nuclei. The inner secretory substance varies considerably in appearance. In some cells it is merely granular, in others it shows comparatively large globules, and sometimes quite large vacuoles.

In all the complete and undistorted sections the glandular mass is definitely separated from the chitin of the side opposite to the spinneret-spines by a space containing a finely granular substance (green in the section) which has all the appearance of insect blood after treatment with a fixative. There seems little doubt that there is a large blood-space in this position, and that its pressure, increased by some contractile action in another part of the body, serves to control the exudation of the silk from the spinneret-spines.

Although not showing continuously in any of my sections, I am inclined to think that the proximal ends of the glandular cells lie upon, or perhaps by their fusion form, a delicate membrane lying between them and the blood-space. The diameter of the gland-ducts is about 0·002 mm. Fig. 5 shows a view under the 1/12 inch oil-immersion objective of a single spinneret-spine of *H. quadrivittata* in its socket, with the gland-duct joining it, and part of the cell from which the duct arises. The delicate membranous " valve " can be seen at the end of the spine. The spine itself is about 0·025 mm. in length and about 0·0035 mm. in diameter, although thicker at the base.

Examples of the production of silk by a mature insect are of the greatest rarity. Silk is produced by species of Hydrophilinæ (*Coleoptera*) which make egg-cocoons. Psocids cover their eggs with little silken pads or scales. Embiidæ live in silken galleries. After I had made the foregoing observations on Hilara, Mr. O. W. Richards kindly called my attention to a paper I had not seen,* in which Melander describes the silk-producing glands in *Embia texana*, Mel., and shows that the silk from this species is also produced " in the thickened anterior metatarsi, within chambers,† and conducted outside to the recurved hairs at the edge of the plantar surface."

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* Melander, *Zool. Bull.*, vol. 3, p. 16 (1902).

† Melander uses the word " chambers," probably assuming that each silk-gland is multicellular, as indeed they may be in Embia. Those in Hilara are, in my opinion, unicellular.

Melander gives a diagrammatic section of the part of the tarsal joint of *E. texana*, and though differing in detail, the apparatus is in general arrangement similar to that found in Hilara. That silk-glands should be found in the tarsal joints of insects belonging to two such widely separated orders as Embiidæ and Diptera is probably a unique example of parallel development. I have to thank Mr. Hamm for the whole of the material used in the investigation, and also for valuable assistance. Indeed, without his help and co-operation the work could not have been carried out.

APPENDIX.

On the Epigamic Behaviour of Hilara maura, *Fab., and two Allied Species.*

By ALBERT HARRY HAMM, A.L.S., F.E.S., Assistant in the Hope Department of Zoology, Oxford University Museum.

The following record of observations is offered as a supplement to Dr. Eltringham's researches upon the silk-producing apparatus of the male Hilara.

Hilara maura, Fab.—Around Oxford this species is the commonest and most generally distributed of any in the genus, and is at the same time by far the most interesting because of its extraordinary and remarkably variable habits.

These flies are on the wing from mid-May to mid-June, and in some seasons occur in extraordinary abundance. They are always to be found flying over or near water, although the smaller streams are most favourable for observation. This follows from the fact that the flies collect in incredible numbers on a limited surface, while on rivers or ponds they become so widely dispersed that it is difficult to follow their activities in detail. Hence all my observations have been made on small streams from 3 to 6 feet wide. Sometimes, when one approaches such a stream at the period of maximum abundance, the flies are so numerous that they produce the appearance of a thick haze lying on the water. After rain, the males may sometimes be seen in abundance flying just above the surface of small pools in the roads. I have also seen them in the city itself swarming over the gutters after the passage of a water-cart.

Some of the males are to be seen skimming hither and thither over the water—apparently skating upon it but without touching the surface—and all the time keeping a sharp look-out for any small insect or other object as it comes floating down the stream. Having seized its " prey " a male immediately joins the throng flying from 1 to 3 feet above the water. This swarm, made up of virgin females and males carrying their " prey," flies rapidly for a variable distance along the stream and then returns at a different level, so that there is no interference between the insects passing in opposite directions. The down-stream flight is generally at a lower level. The distance traversed may be as much as 6 yards or over, the limits being sometimes apparently determined by an overhanging branch or other similar partial obstruction.

The males make no apparent choice between the objects on the water, seizing anything and everything that is small and light enough to be raised—minute insects of all kinds, fragments of vegetation, such as bud-scales, etc. At one spot over a small stream, thickly overhung with various trees, numbers of males and paired females were carrying the small, almost colourless globules described on p. 318. The viscid substance of which they are composed, at first believed to be secreted by the male Hilara, is probably produced by Aphides or other Homoptera on the overhanging trees. It is hoped that specially directed observations will decide this question and also show the manner in which the globules are collected by the flies.

The majority of the floating insects probably fall on to the water by accident, or are blown from the overhanging vegetation, and many of them are alive when captured. The objects seized are, evidently by means of a rolling motion, gradually enveloped in fine silken threads, the whole mass eventually assuming a more or less spherical shape. When the object is very minute it is enclosed loosely in a relatively large silken structure, which may assume one of many beautiful forms (figs. 3 to 6, p. 319), and being brilliantly white, is quite conspicuous as it is carried in flight by the males or by the paired females. Some of these " balloons " are empty, but it is possible that the stimulus to spin was provided by some small object picked up but subsequently lost. The " balloons" dropped on the water, either accidentally or when coitus has come to an end, are readily picked up and used over again by other males.

Extensive experiments, made over a number of years, have enabled me to estimate the time required to construct the silken covering and to perform the other actions which are the necessary antecedents of coitus. It was found possible to stimulate the males at will by strewing on the surface of the water large quantities of both animal and vegetable material, living and dead—Buttercup stamens, ray florets of the common Daisy, Aphides, small gnats, and even partially disabled males of *H. maura* itself. All such objects were picked up with avidity, but after many trials the Daisy florets were found to be most suitable. They floated easily on the surface film, were picked up without difficulty, and, being white, were easy to follow when carried by the flying Hilara. The conspicuousness of the florets and the comparative invisibility of the males bearing them may be inferred from the remark of a little girl standing by the stream when I was experimenting—" Come and see the man who can make the petals fly ! "

These experiments proved that there is great variability in the behaviour of the males. Some of them would pair a few minutes after the floret had been seized ; the majority, however, continued flying in the swarm for a longer period during which the florets were enclosed in silk, and reduced, evidently by rolling and compressing, to a much smaller size. On one occasion the following results were observed. A large number of florets was thrown on the water at 3.10 p.m. ; at 3.20 the net was passed through the swarm in order to collect samples, for the burdens are immediately dropped by the captured males. These florets were found to be considerably attenuated ; but, at 3.30, when fresh samples were taken, they had become still more slender and thread-like, and often much shortened, and were enclosed in silk. These and the results of many other similar experiments may be studied in the Hope Department of the Oxford University Museum.

In another set of experiments the Hilaras were offered partially disabled males of their own species, and these were also picked up from the water, although not with the same ease as the florets. When the object is too heavy to be raised at once from the surface " the male, after seizing it, spins round with great velocity till the load rises on a cone of water and is finally lifted from the apex " (*Ent. Mo. Mag.*, vol. 49, 1913, p. 179). When, as sometimes happens, two or even three males arrive simultaneously at the same object, the ensuing struggle on the surface of the water generally lasts until one or more of the rivals is completely submerged. The vanquished flies are, however, none the worse for the immersion.

The male, when ready to pair, seeks a female from among the swarm, flying below her as he pursues. At the moment of pairing the two insects clinch together, ventral surface to ventral surface, but in an instant the male is uppermost, and the female can be seen in possession of the object previously carried by him. The white " balloons," often spun by the male, are especially easily seen after transference to the female, as are the white florets of the Daisy and yellow stamens of the Buttercup used in my experiments. All these objects are quite conspicuous, hanging from the lower insect of each flying pair, and the lower is invariably the female. The sex can be accepted without doubt, because, at the moment of pairing, the half turn in the air may be seen to bring the male uppermost, and because, when the paired insects are made to alight on the net or in a box, the male is invariably above the female. When thus resting, he intermittently taps the head of the female with the dilated basal joint of the fore-tarsi, moving the legs alternately.

The two insects, as soon as they have paired, fly slowly and steadily away, both sexes using their wings and maintaining a level which is always higher than that of the swarm they have left, and may occasionally reach an altitude of 20 to 30 feet. How long copulation lasts it would be extremely difficult to determine, for the insects never settle but continue their flight for the whole period. Pairs which have been captured and enclosed in a glass-bottomed box remain *in coitû* for several hours.

H. quadrivittata, Mg., is another common species, often found in company with *maura*, although never in anything like the same abundance. In many respects its habits are similar to those of *maura*, although it never spins "balloons" but always binds its "prey" with silk, often enclosing it in a wall of many layers. Repeated experiments have shown that, while the males readily take small insects of many kinds from the surface of the water, they invariably refuse the fragments of plants similar to those accepted by *H. maura*.

H. pilosa, Zett.—The habits of this woodland species were first observed by my friend Mr. O. W. Richards, and a few days later by myself, in June of the present year (1927), in Bagley Wood, near Oxford. The insects were seen flying in the afternoon, about 2 or 3 feet from the ground, under the low branches of a large oak, and at some considerable distance from water. My attention was first attracted by a glistening white object carried by these Hilaras as they flew backwards and forwards. The carriers when taken proved to be males bearing beautiful and remarkable comet-like silken structures (figs. 1 and 2, p. 319), very different from the "balloons" produced by *H. maura*. Paired specimens were also obtained, and in each case one insect—the female it may be safely inferred—was found to be holding a similar silken object. Out of nine such structures carried by males and paired females, taken by us, three enclosed small insects entangled in the silk, while six were without "prey" of any kind.

EXPLANATION OF PLATE 22.

Fig. 1.—A magnified view of *Hilara maura*, male. Note the enlarged basal joints of the fore-tarsi.

Fig. 2.—A more highly magnified view of the enlarged tarsal joint, showing the beads of liquid silk exuding from the spinneret-spines. Owing to the specimen having been partly cleared and viewed by transmitted light it appears browner than in untreated examples.

Fig. 3.—Enlarged view of a "balloon" or "cocoon" made by *H. maura*. It contains a small particle apparently of some vegetable débris.

FIG. 4.—Magnified view of a longitudinal section of the enlarged tarsal joint of a male of *H. quadrivittata*, as it appears when stained with eosin, methyl-blue, orange-G, and light-green. The gland-cells have a pink cytoplasm enclosing a mauve substance which is the silk secretion, and each cell has a delicate tube leading to a spinneret-spine. The tubes can, of course, only be seen when they happen to lie in the plane of the section. To the right is a granular mass stained green, and doubtless representing blood, by the pressure of which the secretion of the glands is expelled.

FIG. 5.—A highly magnified view of one of the spinneret-spines in its socket, with the tube and part of its respective gland cell. Note the delicate membranous extremity of the spine.

FIG. 6.—A small fly (*Hilara* sp.) which after being disabled and thrown on the water was picked up by a male of *H. maura* and bound with silk.

A Further Induction of Melanism in the Lepidopterous Insect, Selenia bilunaria *Esp., and its Inheritance.*

By J. W. HESLOP HARRISON, D.Sc.

(Communicated by E. W. MacBride, F.R.S.—Received November 17, 1927.)

I.—*Introductory.*

The subject of the experimental induction of melanism in the Lepidoptera, by feeding the larvæ of the species employed on food artificially charged with manganese or lead salts, has already been discussed in a former paper (Harrison and Garrett, 1926). However, as was definitely indicated in the concluding sentences of that contribution, the work left it quite an open question whether one had to look upon the metal or the acid radical in the compound supplied, as the active agent in bringing about the induction. Recognising this, we stated that further researches were contemplated on similar lines at an early date, to settle the matter.

A few months after the conclusion of the earlier work I came into possession of an adequate supply of pupæ of *Selenia bilunaria* (the Early Thorn), collected in the mountain district between Saxony and Czechoslovakia, and I determined to commence the work at once, restricting the investigation to the effects of the metal manganese. Since the manganese salt used in the former investigation had been the sulphate, in the newer experiments recourse was made to the chloride.

Furthermore, for certain reasons, the methods of administering the compounds formerly adopted were abandoned in favour of those used in the still earlier preliminary work of 1917 (Harrison, 1926). Instead of supplying the larvæ with cut twigs which had been charged with metallic compounds by allowing their cut ends to stand for 24 hours in a 0·01 per cent. solution of the salt, the foliage introduced into the cages was freely sprayed with a 1 per cent. solution. By choosing this method a much closer approximation was made to the conditions of food-plants in industrial districts, and at the same time it was possible to keep the twigs in the cages standing in ordinary water.

Needless to say, except under conditions of emergency mentioned below, no change was made in the choice of food-plant, hawthorn (*Cratægus Oxyacantha*) being uniformly supplied on account of the ease with which it is manipulated under cage conditions.

II.—*The Experiments.*

The pupæ received, which were of course of wild origin, were gently forced in order that an early start could be made with the experiments. In consequence, the bulk of the insects emerged in January and February.

As is well known, *Selenia bilunaria* is both seasonally and sexually dimorphic, so that the imagines thus bred were necessarily the heavily marked larger insects of the spring[*] generation. Nevertheless, it should be noted that, although the distinguishing features are not great, they can be differentiated racially from the corresponding generation of British origin.

From this batch a single pair, emerging in January, gave rise to the ova on which the work was based. These eggs, 141 in number, were kept in a fairly warm room, so that just as with the parent imagines, the emergence of the larvæ was accelerated. Only 9 eggs proving infertile, 132 larvæ were obtained, and immediately divided into two lots, each comprising 66 individuals. One of these batches served as a control and was supplied with untreated food, whilst the other was fed on contaminated leaves.

Until they had reached the end of the second instar, both lots were kept in shallow, air-tight, glass-topped boxes, 3½ inches in diameter, in which the twigs of the food-plant were inserted without water. During the early stages of the third instar they were transferred to cylindrical glass cages containing the cut twigs of hawthorn standing in water. So speedily did the larvæ, both control and treated, develop that, by the end of March and the beginning of April, new batches of imagines, with the facies of the summer brood *juliaria*, were in the cages.

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[*] It will thus be clear that the present work, unlike that described in the 1926 paper, commenced with the spring emergence and not with the summer brood to which Haworth applied the name *juliaria*.

In the case of the controls there appeared 28 females and 23 males, whilst the experimental lot yielded 19 females and 27 males. In the latter case, a single pair was chosen with which to continue the work; on the other hand, in order to render the control as rigid as possible, the progeny of three pairs was reared to provide the next generation in that set. This, the second inbred lot in both series, control and otherwise, succeeded just as expeditiously for it appeared in May.

Once more the whole of the insects reared were of the usual summer *juliaria* type, and, again, to continue the work, a procedure similar to that adopted with the second brood of the year was followed. A single pair selected from the treated brood sufficed to continue that series, whilst three pairs served in a like capacity for the controls, although, as will be seen from the tables, in the latter case the two parents of any given control batch were not necessarily taken from the same control family.

Quite unexpectedly, the fourth brood of larvæ in both sets fed up rapidly and produced imagines, again of the *juliaria* form, in September. Again, the selection of parents was made as in the previous generation, and their offspring as larvæ provided in their early instars with hawthorn as food. However, very soon, owing to the lateness of the date, difficulties were encountered in procuring food, for the foliage of hawthorn is soon rendered unsuitable for food purposes. The food-plant was therefore compulsorily changed, firstly, to alder (*Alnus rotundifolia*) and, finally, to bramble (*Rubus fruticosus* agg.). In spite of these changes, and the unusual time of year, pupæ were successfully obtained.

Here, however, the two series diverged in behaviour. The control pupæ remained undeveloped until March; the treated lot, on the contrary, began to emerge on December 6, and by December 19 three females, including two types and one melanic specimen, were in the cages. The experiments had thus once more ended in success.

As no males of any form synchronised in appearance with the first melanic female, it proved useless as far as heredity tests were concerned. In January, however, a second melanic female was bred and, very fortunately, mated with a precocious typical male of Sussex origin. From this crossing, imagines were in due course reared and are listed in Table III as family *a*.

Here it is well to emphasise that up to this time the whole of the insects bred in the treated family T_4 had resembled the summer generation *juliaria*. Subsequently the brood straggled out until well into April, during the whole of which period the moths emerging, whether types or melanic,* were of the spring form.

In all, four melanics (three females and one male) were secured, but, in addition, there were four curiously marked mosaics, including two females and two males. One of these males is radially streaked on the left forewing and right hindwing with a deep blackish purple pigment, whilst a second bears a similar decoration on the left hindwing as well as on the same two wings. The two females carry their additional markings on much more limited areas and only on one wing—the left hindwing. However, the depth of coloration, in both instances, is so great as to appear quite black by contrast with the pale ground colour of the rest of the wing.

The better marked female specimen bears radial marks on the anal two-thirds of the wing, with the streaks so close together towards the margin outwardly as to give the impression of blotches rather than of streaks. The fourth insect, which was not at first recognised as a mosaic, bears two narrow wedges near the anal angle, the inner one being 1 mm. in length and the outer one 3 mm. In addition, at the end of vein 5 can be discerned a small black spot.

Returning now to the history of the broods, the controls have been continued, and with the same number of broods in the cages at any given time as before until the present time, with the result that four additional generations and, therefore, 12 further broods have been reared. Never at any time, as Table I indicates, has there been any deviation from the normal composition of the broods, types and only types appearing.

Owing to the prolonged periods of eclosion of the treated brood, only one pairing of its types *inter se* was possible. This, in due course, gave rise to larvæ likewise subjected to treatment with food sprayed with manganese chloride in 1 per cent. solution. Imagines were secured in April, and these included a huge preponderance of melanics; indeed, of a batch comprising 29 individuals (14 females and 15 males), only four moths (two females and two males) were typical. These types were so far apart in time of appearance that further pairings within the limits of the treated strain proved impossible.

As with the one of the melanics in the parent brood, two of the melanics in this brood (T_6) were outcrossed with types; in one case a type from control batch C_{12} was introduced and in the other an unrelated Saxon male. The two F_1 lots so obtained are those labelled family *c* and family *b* respectively.

To describe the further experiments designed to test the inheritance of the induced melanism would be tedious and confusing, and, moreover, quite unnecessary. The observations have therefore been thrown into the form of Tables III, IV, V, VI, VII and VIII.

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* Exactly as in the case of the types the melanics are seasonally dimorphic.

Table I.—Control Broods.

Family.	Origin of family.	Females.	Males.	Facies of imagines.	Principal period of emergence.
C	Wild origin	28	23	Typical *gen. aest. juliaria*	March.
C_1	C ♀ × C ♂	63	58	,, ,,	May.
C_2	C ♀ × C ♂	51	49	,, ,,	
C_3	C ♀ × C ♂	41	57	,, ,,	
C_4	C_1 ♀ × C_1 ♂	48	36	,, ,,	July.
C_5	C_1 ♀ × C_3 ♂	53	51	,, ,,	
C_6	C_3 ♀ × C_2 ♂	54	56	,, ,,	
C_7	C_4 ♀ × C_4 ♂	38	29	,, ,,	September.
C_8	C_5 ♀ × C_6 ♂	36	51	,, ,,	
C_9	C_6 ♀ × C_5 ♂	42	48	,, ,,	
C_{10}	C_9 ♀ × C_9 ♂	46	49	Typical *gen. vern.*	February to April.
C_{11}	C_7 ♀ × C_8 ♂	26	29	,, ,,	
C_{12}	C_8 ♀ × C_7 ♂	33	38	,, ,,	
C_{13}	C_{10} ♀ × C_{12} ♂	59	72	Typical *gen. aest. juliaria*	May.
C_{14}	C_{11} ♀ × C_{11} ♂	65	63	,, ,,	
C_{15}	C_{12} ♀ × C_{11} ♂	38	29	,, ,,	
C_{16}	C_{13} ♀ × C_{14} ♂	37	55	,, ,,	July.
C_{17}	C_{14} ♀ × C_{13} ♂	49	56	,, ,,	
C_{18}	C_{15} ♀ × C_{15} ♂	31	33	,, ,,	
C_{19}	C_{16} ♀ × C_{18} ♂	47	48	,, ,,	September to October.
C_{20}	C_{17} ♀ × C_{17} ♂	48	42	,, ,,	
C_{21}	C_{18} ♀ × C_{16} ♂	39	34	,, ,,	
	Totals	972	1006		

Table II.—Treated Broods.

Family.	Origin of family.	Females.		Males.		Principal period of emergence.
		Types.	Melanics.	Types.	Melanics.	
T	Wild	19	—	27	—	March.
T_1	T ♀ × T ♂	49	—	53	—	May.
T_2	T_1 ♀ × T_1 ♂	42	—	36	—	July.
T_3	T_2 ♀ × T_2 ♂	38	—	35	—	September.
T_4	T_3 ♀ × T_3 ♂	27	3 + 2 mosaics	26	1 + 2 mosaics	December to April.
T_5	T_4 ♀ × T_4 ♂	2	12	2	13	April.
	Totals	194		195		

Table III.—Homozygous Type × Homozygous Melanic.

TT × *tt*.

Family.	Origin of family.	Types.		Melanics.	
		Females.	Males.	Females.	Males.
a	Melanic T_4 ♀ × type Sussex ♂	31	37	—	—
b	Melanic T_5 ♀ × type Saxon ♂	45	34	—	—
c	C_{12} ♀ × melanic T_5 ♂	38	42	—	—
n	*d* ♀ × C_{18} ♂	53	54	—	—
o	C_{17} ♀ × *e*	49	61	—	—
p	C_{16} ♀ × *d* ♂	43	49	—	—
	Actually reared	536		0	
	Expected	536		0	

Table IV.—Heterozygous Type × Homozygous Black.

T*t* × *tt*.

Family.	Origin of family.	Types.		Melanics.	
		Females.	Males.	Females.	Males.
f	*a* ♀ × black ♂ *ex* EL*	24	23	21	29
g	*a* ♀ × black ♂ *ex* EL	29	27	20	23
k	*b* ♀ × *d* ♂	19	19	17	18
l	*d* ♀ × *b* ♂	16	18	14	23
m	*d* ♀ × *b* ♂	28	24	19	27
	Actual result	227		211	
	Theoretical result	219		219	

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* "*Ex* EL" indicates that the insect so designated is a direct descendant of the family 25 EL of the 1926 paper.

Table V.—Heterozygous Type × Heterozygous Type.

T*t* × T*t*.

Family.	Origin of family.	Types.		Melanics.	
		Females.	Males.	Females.	Males.
d	*a* ♀ × *a* ♂	36	38	15	12
e	*a* ♀ × *a* ♂	8	5	3	2
q	*b* ♀ × *b* ♂	28	28	8	11
r	*c* ♀ × *c* ♂	31	32	9	9
s	*b* ♀ × *c* ♂	17	12	6	3
v	*r* ♀ × *r* ♂	1	6	2	3
w	*r* ♀ × *n* ♂	19	23	4	7
x	*n* ♀ × *i* ♂	28	30	9	10
y	*s* ♀ × *s* ♂	35	42	12	16
z	*d* ♀ × *d* ♂	32	25	14	12
	Actual result	476		167	
	Theoretical result	482·25		160·75	

Table VI.—Homozygous Black × Homozygous Black.

tt × *tt*.

Family.	Origin of family.	Types.		Melanics.	
		Females.	Males.	Females.	Males.
t	*d* ♀ × black ♂ *ex* EL	—	—	25	23
u	*d* ♀ × black ♂ *ex* EL	—	—	42	47
A	black ♀ *ex* EL × *d* ♀	—	—	18	29
B	*d* ♀ × black ♂ *ex* EL	—	—	22	28
D	*d* ♀ × *d* ♂	—	—	8	4
	Actual result	0		246	
	Theoretical result	0		246	

Table VII.—Homozygous Type × Heterozygous Type.

TT × T*t*.

Family.	Origin of family.	Types.		Melanics.	
		Females.	Males.	Females.	Males.
h	Surrey ♀ × *a* ♂	17	20	—	—
i	*a* ♀ × Surrey ♂	43	48	—	—
j	*a* ♀ × Surrey ♂	31	36	—	—
a'	*b* ♀ × Surrey ♂	19	27	—	—
b'	Surrey ♀ × *b*	28	34	—	—
	Actual result	303		0	
	Theoretical result	303		0	

Table VIII.—Either Extracted Homozygous Type × Heterozygous Type or Extracted Homozygous Type × Extracted Homozygous Type.

TT × T*t* or TT × TT.

Family.	Origin of family.	Types.		Melanics.	
		Females.	Males.	Females.	Males.
E	*d* ♀ × *d* ♂	42	48	—	—
F	*d* ♀ × *d* ♂	37	39	—	—
G	*d* ♀ × *d* ♂	38	61	—	—
	Actual result	265		0	
	Theoretical result	265		0	

From these every essential fact may be determined, and the whole of the evidence they afford amply confirms the deduction from the earlier researches, that the melanism developed in *Selenia bilunaria* is inherited on a Mendelian basis as a simple recessive.

A few remarks must now be introduced in connection with the mosaics. Naturally, it was of extreme importance that their germinal condition should be tested. To do this the most strikingly marked male was mated with a type Surrey female, as a result of which an F_1 brood of 49 typical insects (20 females and 29 males) was reared. Of these, six couples were placed together in a pairing cage, and about 850 fertile eggs secured, from which a random selection of 100 was made to procure the F_2 lot. These gave rise to 78 moths (36 females and 42 males) which again were all typical in appearance. Clearly, enough pairs were taken to make it practically certain that at least one of the matings should be type heterozygous female × heterozygous male, had the mosaic parent been the unusual expression of a heterozygous insect. Hence, in the absence of melanics in the mixed F_2 lot, one seems compelled to assume that the mosaics arose as the result of a fairly early localised induction restricted to the soma.

I need hardly mention that, during the whole of the investigations dealing with the inheritance of the melanism, the larvæ were uniformly supplied with pure food.

III.—*Concluding Remarks.*

Very little comment seems necessary in connection with the above results. For a third time, by suitable treatment, melanism has been induced in a strain of *Selenia bilunaria*, known to be quite free from inherited melanism, not only from the evidence of a large number of consecutive control generations but also from the fact that it was brought from an area in which that insect is quite typical. Moreover, this area in Eastern Saxony is far removed from Sussex which provided the material used in the former work.

Further, additional breeding operations, undertaken with the induced melanism, have proved definitely that the matter is not one of a mere somatic induction, but that the melanism has, so to speak, been registered in the germ plasm; without doubt the melanism is inherited as a Mendelian recessive.

No claims are made here or elsewhere that we are concerned with a Lamarckian effect. Coupling the work described above with that discussed in the 1926 paper (Harrison and Garrett), it is almost certain that the result has been produced by the direct action on the germ plasm of the metallic compound administered with the food. This, as I have already pointed out (Harrison, 1927 *a*, Harrison, 1927 *b*), affords a new principle in evolution and would suffice to account for evolutionary progress in a variety of forms, both animal and plant, in Nature and in the hands of man.

When the earlier results were announced a statement was made that melanic forms, such as had been developed, had not been captured, or observed, wild. Shortly after the publication of the paper, Cockayne announced to me in a letter, and later (Cockayne, 1927) published a note to the effect, that melanic specimens of *Selenia bilunaria*, similar to those obtained experimentally, had been reared from a wild female taken in the West Riding of Yorkshire—notoriously a district in which melanism of the so-called industrial kind seems most prevalent.

In view of the possibility of criticism in this direction, it should be made clear that the present work is not regarded as solving the problems of industrial melanism, although it is claimed that some light has been thrown on the matter. This seems the more certain when it is made known that a member of the staff of Leeds University informed me recently that the stones in many of the streams near Leeds, where melanism prevails, were covered with a thin coating of a mud composed of manganese compounds.

Only one point remains for treatment, and that is to determine to what extent an answer has been obtained to the question originally propounded when the work commenced :—Is the responsible agent in the induction the manganese or the acid radical ? At first sight the answer seems to be the perfectly obvious one that, since the manganese remained constant in the two experiments whilst the acid radical varied, the metal is the inciting agent. Nevertheless, the possibility suggested by Poulton, that the phenomenon may be one of "shock," capable of being brought about by many agents, must not lightly be cast aside.

IV.—*Summary.*

1. By administering food containing manganese chloride to a strain of *Selenia bilunaria*, known by the use of adequate controls to be free from heritable melanism, melanic insects have been developed.

2. This melanism has been proved to be inherited as a Mendelian recessive.

3. Certain mosaics were obtained in the critical treated brood but these, from experimental tests, seem to represent cases of induction restricted to limited areas in the soma, the germ plasm being apparently unaffected.

4. The effect is not of the Lamarckian type, but rather illustrates a new evolutionary principle, that heritable variations may be induced by means of the food supplied.

5. Although other explanations are not absolutely excluded, the experiments

indicate that the metal is the active agent in bringing about the observed effect.

I should like to acknowledge here the receipt of a grant of £15 in aid of this and other researches from the British Association.

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Induced Changes in the Pigmentation of the Pupæ of the Butterfly Pieris napi *L., and their Inheritance.*

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(Communicated by E. W. MacBride, F.R.S.—Received November 18, 1927.)

I.—*Introductory—Materials, Method, etc.*

It has, of course, long been known that the pigmentation of the pupæ of the two lepidopterous species, *Pieris brassicæ* L. and *P. rapæ* L. (Poulton, 1887), is influenced by the colour of the light to which the larvæ from which they are developed are exposed just before pupation. In particular, if the larvæ are allowed to pupate in orange light, a heavy percentage of the pupæ obtained is of a bright green colour. This coloration, however, simply depends upon the fact that the formation of the various pigments, white and black, in the chitin of the pupal integument is more or less inhibited by orange light. As a consequence, the colour perceived in pupæ so affected is that of the hæmolymph within, and hence the chrysalids appear* green.

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* Analogous but not the same effects are brought about when the larvæ pupate under blue and other glasses.

Recently, in addition to confirming these earlier observations, Dürken (1923) and Brecher (1923) have bred from butterflies reared from the treated larvæ and pupæ. The larvæ descended from the orange glass cultures, even when pupating under ordinary light conditions, gave rise to a set of pupæ including many more green individuals than the control lots. Clearly, therefore, the acquired pupal green had been inherited. In view of current biological controversies, such an effect was of primary importance. Repetition and confirmation of the work were highly desirable, and the present work was therefore undertaken.

Recognising that it was preferable to test the matter with other species, I determined to avail myself throughout the work of *Pieris napi*, a species related (though not so closely as the use of a common generic name would imply) to *P. brassicæ*. An additional gain secured by the use of the related species was the accumulation of facts in respect to its reaction, at the critical moment before pupation, towards coloured light.

The cages provided for the pupating larvæ were specially built, of size 12 inches × 12 inches × 9 inches, glazed on the four sides and the top. Further, they were designed to be as nearly as possible air-tight, and illuminated thereore solely by the light transmitted by the glass. Of such cages nine were constructed, but, in the end, only four were brought into use.

After very careful tests, the glass in the cage intended to subject the larvæ to orange light was deemed to transmit too much blue. To cut this out, the glass was painted outside with a strong solution of a yellow dye, with a decided tendency to orange, but of unknown chemical composition. This solution was rendered more adhesive by the addition of large quantities of sugar. Similarly, the floor and woodwork of the cage inside were painted with an aqueous solution of the same dye.

Since these cages were air-tight, my original intention was to utilise them for pupation purposes only, and to transfer the larvæ to them towards the end of the last instar. Direct experiment, however, proved that larvæ were quite tolerant of the conditions within, and they were subsequently used as breeding cages. As they were obviously too large for newly hatched larvæ, the leaves bearing ova were always placed with a supply of food-plant in 3½-inch glass-topped tin boxes, which were in turn placed within the cages. From these the larvæ, as soon as they reached the fourth instar, were allowed to wander, to reach cut portions of the food-plant standing in water.

Hence, although prolonged exposure to the experimental light was known to be quite unnecessary, from the moment the larvæ hatched to the time the imagines emerged, the insects were subjected to the influence of the coloured light chosen. Moreover, as the period for pupation approached, except in the case of the control cages fitted with colourless glass, new food supplies were given, and the cages cleaned out, in a photographic dark-room lit by a dim ruby light. One further precaution taken must be mentioned, and that is that, to secure even illumination, the cages were kept standing out of doors, whenever the weather permitted, in bright diffused light and on sheets of white paper.

These cages, even those provided with ordinary window glass, are of no value for pairing the insects. Butterflies are in any case much more difficult to mate than moths, and, to secure the best results, bright sunshine and a free current of air are essential. Thus, for pairing purposes, special cages had to be made. Twelve lengths of wood, 1 inch square in cross-section and 15 inches long, were nailed together, so as to form a cubical framework, which, except on one face, was covered with fairly stiff "book" muslin. This muslin was secured by glueing it down wherever it touched the wood.

A flat board was next taken on which was placed a jar containing the food-plant. The cage was then put over it and the insects inserted, and all taken out of doors into the sunshine. Under such conditions *P. napi* pairs readily and lays its eggs freely; no difficulties,[*] such as Dürken reports for *P. brassicæ*, being encountered in connection with either operation. The only further precaution to be observed is to spray the muslin and the food-plant occasionally with water, or, if it is preferred, to insert regular supplies of newly expanded dandelion flowers.

A word of warning is necessary in the matter of a suitable food-plant. In my researches, although I recognised that the normal food-plants of *P. napi* in nature are watercress (*Nasturtium officinale*) and cuckoo flower (*Cardamine pratensis*), I elected to use charlock (*Brassica arvensis*). This the larvæ accepted with avidity. Not only was this so, but, in addition, they developed speedily and well. Only when I came to secure ova from their progeny were its defects revealed, for I discovered that under its influence the insect becomes barren, the actual number of eggs laid diminishing enormously as well as their fertility. Should I repeat the experiments, in the absence of abundant supplies of watercress or cuckoo flower, I shall certainly avail myself of garden mignonette* (*Reseda odorata*) which the species chooses when it deserts its favoured swamps and damp meadows for gardens.

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[*] Oviposition will proceed with this species, should dull weather intervene, quite satisfactorily when the cages are exposed to electric light or incandescent gas light. For all of these reasons *P. napi* is recommended to future workers desiring to employ a butterfly for experimental work; only *Brenthis dia*, *Pararge ægeria* and *P. megæra* equal it in such respects.

II.—*The Experiments.*

To commence the work six wild females of *Pieris napi* were received from Stewartstown, Co. Tyrone, Ireland, but these, arriving after prolonged delay, during the General Strike of 1926, were so exhausted that instead of isolating them in separate cages I had simply to feed them and transfer them to one large cage for oviposition. Thus, the eggs secured represent the mixed batches from six females.

In all, 255 ova were deposited and divided into three equal lots. One of these was reared in the cage (cage C), provided with ordinary window glass to serve as a control, whilst the second and third were placed under blue (cage B) and orange (cage O) glass, respectively. Of these three sets, that under the blue glass gave rise to the greatest number of pupæ, 73 in all being obtained. Under the orange glass 46 were reared whilst the control provided 43.

Classified according to pigmentation the pupæ gave the following results :—

(A) The controls—cage C.

- (*a*) 34 waxy white in the ground colour, but with their angulations varying in depth of pigmentation from that represented for *Pieris rapæ* on Poulton's plate 26 in fig. 37† to that shown on fig. 32. These two varied in the amount of grey shading on the wing cases.
- (*b*) 5 were greenish but intermediate between figs. 39 and 40, whilst the pigment on the angles varied as with the whiter ones.
- (*c*) 4, except that they displayed a slight blackening on the angles, were like fig. 41.

Summing classes (*b*) and (*c*) together the percentage of green pupæ amounts to 20·9, although the difference in depth of coloration between the two classes (*b*) and (*c*) should be noted.

(B) Under orange glass—cage O.

- (*a*) 1 nearly pure white.
- (*b*) 1 like the *P. rapæ* represented by fig. 39.
- (*c*) 1 pinkish and resembling fig. 37.
- (*d*) 43 clear green with the angles only slightly pigmented.

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* On the Fife coast I have found many wild colonies attached to *Reseda lutea* and *R. luteola*.

† The references to the figures are all to those of *P. rapæ* on Poulton's (1887) plate 26.

In this case the percentage of green examples attains the high level of 93·4.

(C) Under blue glass—cage B.

- (*a*) 12 similar to fig. 32.
- (*b*) 19 like fig. 33.
- (*c*) 24 distributed amongst figs. 34, 35, 36, and 37.
- (*d*) 2 whitish.
- (*e*) 14 green but heavily marked with black as in fig. 32.
- (*f*) 2 green but a little more heavily marked than fig. 40.

Here, adding the strongly pigmented greens of class (*d*) to those in (*f*), the percentage of green pupæ is 21·9—a figure exceedingly close to that revealed for the controls.

At this stage, chiefly for lack of cage space, the cultures from the blue glass cage were discarded. On the other hand, the whole of the insects emerging in the control cage were placed in a pairing cage, and ova obtained, a similar course being pursued, after the removal of the non-green pupæ, with those coming out under orange light. The controls were reared as before to yield pupæ, not differing significantly in the proportions assigned to the various colour classes, from the previous lot. Similarly, the progeny of the orange glass cultures, once more reared under orange glass, gave rise to an enormous preponderance of green pupæ, 95·2 per cent. being of that colour.

Here the control strain was abandoned but the work was continued with the orange glass series after the elimination of the non-green pupæ. The resulting imagines were successfully mated; however, instead of rearing their progeny under orange glass, they were placed in the control cage, where in the end, when allowed to pupate in ordinary daylight, they yielded 31 pupæ—all green. This striking result was wholly unexpected for, if due weight was to be attached to the cultures preceding it, the appearance of a small number of pupæ of different pigmentation was to be anticipated.

The butterflies bred from these seemed to break into two batches—one of immediate, and the other of delayed, emergence. The earlier batch, already exhibiting diminished fertility, nevertheless provided enough ova to continue the experiment for a further generation. This, to make the tests as searching as possible, I determined to rear and to pupate in a cage offering as varied a selection of surfaces for pupation as possible.

An account of the colours, with other necessary details concerning the pupæ, is appended.

(1) On red tile.
  (*a*) 1 white dark marked.

(2) On glass.
  (*a*) 1 very dark (Poulton, fig. 33).
  (*b*) 7 green (fig. 40).
  (*c*) 4 light green (fig. 39).

(3) On perforated zinc.
  (*a*) 1 blackish (fig. 32).
  (*b*) 2 greyish (fig. 35).

(4) On top, mottled grey and white.
  (*a*) 4 whitish.
  (*b*) 5 marking less white.
  (*c*) 6 ranging between Poulton's figs. 33 and 36.
  (*d*) 14 green.

(5) Loose on bottom.
  (*a*) 4 green.
  (*b*) 1 greyish.

In this instance the percentage of green pupæ was 58.

When the latter part of the batch which provided the culture just described came out,* it likewise yielded a supply of ova far inferior, both in actual numbers and fertility, to those from wild females. Still, I was able to obtain 40 larvæ. These, instead of being reared in cages such as were employed previously, were placed in one exactly like cage C, except that the glass was painted black. Thus, the larvæ were reared wholly in the dark, under which treatment they yielded 21 pupæ, 26 green and 1 greyish, or a percentage of 96·4 green pupæ.

Taking due cognisance of the conditions of this brood and of the two just discussed, there can be no doubt but that the green pupal colour, induced by the inhibition of pigment by the use of orange light, is inherited, *no matter how.*

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* At this stage I reared another wild brood of *P. napi* of Scottish (Fife) origin in a cage similar to cage C. This produced 45 non-green and 12 green pupæ—or 21 per cent. of green examples. This is almost exactly the same figure as in the case of the wild Irish ones and confirms, not only the actual numerical results, but also the observation that, in the case of *P. napi*, the normal figure for green pupæ is distinctly greater than in *P. brassicæ* or *P. rapæ*.

In other words, ample confirmation has been obtained of Dürken's and of Brecher's work.

An attempt was made to proceed further with the work, but, after sacrificing the whole of the imagines obtained in the last batch but one, only six fertile ova were deposited, and the investigation thereby compulsorily brought to a close.

III.—*Summary.*

1. The pupæ of *Pieris napi*, when developed from larvæ exposed at the critical time to lights of different colours, are influenced in their pigmentation like those of their congeners *Pieris brassicæ* and *P. rapæ*.
2. As Dürken and Brecher found in the case of *P. brassicæ* the green pupal colour, acquired under the influence of orange light, is inherited.
3. The work of those two investigators is therefore confirmed.

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Immunological and Serological Studies on the Viruses of Fowl-Pox and Vaccinia.

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(Communicated by J. A. Murray, M.D., F.R.S.—Received December 3, 1927.)

CONTENTS.

	Page.
General Introduction	354
Material	355
Fowl-pox—the natural and experimental disease	356
The Calmette-Guérin phenomenon	357
The potency of fowl-pox and vaccinia viruses	359
The development of natural immunity to fowl-pox	361
The duration of immunity to fowl-pox virus	364
The serology of fowl-pox	365
The relationship between the viruses of fowl-pox and vaccinia	371
The filterability of vaccinia and fowl-pox virus	375
Conclusions	377

General Introduction.

Of the many problems in medicine which still await solution none are of more importance than those connected with the group of diseases now recognised as due to ultra-microscopic viruses. While the study of bacterial diseases has made continuous progress, our knowledge of infections due to ultra-microscopic viruses has remained relatively stationary, as a result of the difficulty in determining the presence of these viruses apart from experiments on the living animal and of the impossibility of cultivating them on artificial media.

At the present time it is obvious that fundamental advances can only be made by intensive studies of the more easily accessible viruses, and in the investigations here detailed attention has therefore been concentrated on the virus of fowl-pox, or epithelioma contagiosum, and the virus of vaccinia. The virus of fowl-pox has certain special advantages for an intensive study, in that it occurs in various species of domestic birds, it is filterable, and in addition it belongs to that great group of ultra-microscopic organisms which comprises not only the virus of vaccinia but those also of variola, sheep-, goat- and horse-pox. The relationship between the members of this group has been a subject of continued interest since Jenner (1798) described in his classical experiments the protective capacity of the virus of cow-pox against that of variola.

The main object of the present research has therefore been to determine the relation between fowl-pox and vaccinia, though before that problem could be attacked it was necessary to have some clear idea of the relationship between the various strains of the fowl-pox virus itself.

Material.

Three strains of fowl-pox virus were available for the present investigations.

Strain A was derived from a spontaneous epidemic occurring in hens on a poultry farm near St. Albans, Hertfordshire.

Strain B was obtained, through the kindness of Dr. R. A. O'Brien, from the Wellcome Physiological Laboratories, Beckenham, Kent.

Strain C was forwarded from Florence by Dr. Lusena, to whom our thanks are due. This strain was obtained from an epidemic in pigeons and was that originally used by van Heelsbergen (1920) in Holland.

A specimen of a partridge *Perdrix cinerea* suffering from the disease was also sent to us by the kindness of Dr. S. P. Bedson of the London Hospital. Unfortunately this strain failed to infect either pigeons or hens.

A supply of raw calf lymph was obtained through the kindness of Dr. Green of the Lister Institute Lymph Establishment, Marazion, Cornwall. The experimental animals used were hens, pigeons, ducks, rabbits, rats and calves. For the routine transmission under laboratory conditions of the virus of fowl-pox, chickens and pigeons were used, while for the virus of vaccinia, chickens and rats were employed.

Chickens, from one day to a week old, were found to be much more uniformly susceptible than older hens to the viruses of both vaccinia and fowl-pox, and were therefore used throughout the experiments.

To obtain material for routine transmission either from fowl-pox or vaccinia in the chick the epidermal lesions were removed 10 days after inoculation, ground to a powder in a mortar, weighed and diluted with 10 parts of physiological saline solution. The suspension was then centrifuged to throw down gross particles and the supernatant liquid pipetted off. Further dilutions were made as required. In inoculating chicks and pigeons the feathers were plucked from the sides of the head above the eyes and a given quantity, usually 0·05 c.c., of the required dilution of the virus was applied to the bare skin.

In the case of the rat with vaccinia the cornea was lightly scarified with a fine needle and a dilution of the vaccinia virus in saline applied to the scarified surface. The virus was passaged to new animals every six days.

Fowl-pox—The Natural and Experimental Diseases.

In order to clarify much of what follows a very brief account will be given of the clinical and main pathological features of the disease.

Fowl-pox, or as it is frequently called roup or epithelioma contagiosum, is an acute infectious disease occurring commonly in epidemic form among domestic birds, such as hens and pigeons, and more rarely among turkeys and ducks. Epidemics have also been described in wood pigeons, partridges and sparrows. The lesions are generally found on those portions of the head which are destitute of feathers, except in very severe cases, when any portion of the skin, feathered or unfeathered, may be attacked. The conjunctiva, the tongue and the mucous membranes of the mouth and pharynx may also be the site of lesions. In the latter situation a false membrane may be formed, constituting the so-called avian diphtheria, which is now recognised to be identical with fowl-pox. At first the skin lesions appear as small smooth yellowish tumours, which rapidly increase in size, become brown in colour and, if the bird lives, eventually dry up and fall off as crusts without leaving a cicatrix. There are no characteristic changes in the internal organs.

The disease may be experimentally produced by rubbing some of the infective material into a portion of skin from which the feathers have been plucked, or which has been lightly scarified with a fine needle. Two days after inoculation the scarified area is seen to be a little swollen and slightly yellow in colour. The swelling rapidly increases and within four days the area is the site of a small tumour-like growth.

After intravenous injection of a suspension of the virus the lesions appear at the sites of election—the comb, nostrils, corner of the beak, eye-lids or orifices of the ears—after an incubation period of from one to two weeks.

If at the time of injection the feathers are plucked from any area of the body an intensive lesion will develop at this point, a phenomenon similar to that described by Calmette and Guérin (1901) in the rabbit after intravenous injection of vaccinia virus.

The main histological features have been recently described by Ledingham (1924) and consist during the first 24 hours of intense congestion in and swelling of the endothelial cells of the capillaries in the dermis, an exudation of polymorphonuclear leucocytes, and active proliferation of the adventitial cells. At the end of 48 hours, in addition to the dermal reaction, active mitosis in the epidermal cells is noticeable, while many of these cells contain the earliest stages of the virus bodies.

Vaccinia virus when inoculated into the skin or comb of the adult fowl produces only small discrete papules, but in the skin of the young chick there are large yellowish growths which are indistinguishable from those due to the virus of fowl-pox. The greater susceptibility of the young chick to vaccinia virus has also been recently noted by Andervont (1927). In our experiments the incubation period of vaccinia in the chick was always from 8 to 10 days. The histological picture was identical with that of fowl-pox.

The Calmette-Guérin Phenomenon.

As previously noted intravenous inoculation of the fowl-pox virus in a chicken and removal of the feathers from any area of the body is followed 7 to 14 days later by the appearance of a lesion at the site of removal of the feathers. A further analysis of this phenomenon, first described by Calmette and Guérin (1901) in the case of vaccinia virus in the rabbit, throws light on the fate of the virus after intravenous inoculation.

The following experiments were therefore carried out. 0·5 c.c. of a 1 in 10 dilution of the virus was injected into the wing vein of a number of chickens, the feathers having been plucked from the right side of the breast. These chickens were bled at varying intervals after the injection, and the infectivity of the blood tested on other chicks. It was found that the virus had disappeared from the blood stream 6 to 8 hours after injection. Thereafter the blood remained sterile until the eighth or tenth day, when, with the definite appearance of a lesion at the site of injury, the blood and the internal organs became infective and remained so until the twenty-fifth to thirtieth day after the initial inoculation. If during the period that the virus was present in the blood stream feathers were plucked from another area of the body a fresh lesion appeared at this site after an incubation period of from 2 to 4 days. It seems obvious therefore that the re-infection of the blood stream is due to the multiplication of the virus in the epidermal cells of the lesion, since there is no evidence that the blood, *in vitro* at least, is in any way a suitable medium for the multiplication of the virus.

The work of Ledingham (1927) has emphasised the importance of the reticulo-endothelial system in infections with the virus of vaccinia. Ledingham showed that if an area of skin were injected intradermally with an India ink suspension prior to incubation with the vaccinia virus the vaccinia lesion either failed to develop or was very small. Similar results were obtained in the present experiments with the fowl-pox virus in chickens and pigeons.

Areas of skin on both sides of the head of a pigeon were plucked; the area on the left side of the head was inoculated intradermally with a suspension of India ink in saline, the control area on the right side of the head being inoculated with saline only. Four hours later the bird was inoculated intravenously with 0·5 c.c. of a 1 in 10 suspension of fowl-pox virus. No lesion developed on the left side of the head, while a typical lesion developed on the right side.

In a second experiment the intravenous inoculation of the virus preceded by 8 hours the injection of the skin of the head with India ink suspension. In this case the lesion on the left side of the head was only slightly smaller than that on the right side.

In a further experiment two pigeons received three intravenous inoculations of India ink suspension 4 days, 2 days and 1 day before the removal of feathers from the right side of the head and the intravenous injection of the fowl-pox virus. One pigeon showed no lesions and 4 weeks later was not immune to the action of the fowl-pox virus. The second bird 21 days after the inoculation showed a tiny lesion on the upper eye-lid of the right eye. In these two pigeons the blood was tested for infectivity at varying times after injection. The virus was found to be present in the blood stream as long as 36 hours after injection.

The result of these experiments therefore goes to prove that following intravenous inoculation of the virus, the reticulo-endothelial system removes the virus from the blood stream, since in birds in which the reticulo-endothelial cells are blockaded with India ink the virus remains in the blood stream longer than normally and eventually disappears without infecting the epidermal cells.

The question however still remains as to the reasons why, after intravenous injection of the virus, the lesion should appear at the site of injury. Levaditi (1922), in dealing with the Calmette-Guérin phenomenon, has made the suggestion that the virus only enters the epidermal cells when they are undergoing mitosis. Since the actual entry of the virus into the epidermal cell cannot be seen under the microscope it is not easy to deny this possibility. The following considerations and experiments however render Levaditi's explanation unlikely. Hance (1927) has shown that after shaving the hair of the mouse the cells in the hair follicles do not begin to undergo mitosis until 48 hours after the injury, the maximum number of mitoses being found 8 to 12 days after the depilation. So far as our observations on chick skin go, there appears to be a similar interval after plucking before mitosis begins in the epidermal cells. If Levaditi's theory is correct inoculation of the virus 8 to 12 days after plucking should give a rapidly growing, massive reaction at the site of injury, since the actively dividing epidermal cells should be peculiarly liable to infection by the virus. Intravenous inoculation of the virus 8 days after plucking is not, however, followed by any lesions at the site of the injury; inoculation of the virus 4 days after plucking produces a small lesion, while intravenous inoculation of the virus 2 hours after plucking, when as yet there is no mitosis of the epidermal dells, produces a massive rapidly growing reaction. There is thus no evidence to confirm the suggestion that the cells of the epidermis when undergoing mitosis are more liable to infection by the virus. The damage to the capillaries of the dermis, and the congestion of these vessels following the forcible removal of the feathers, would appear to be the main factors in causing the accumulation of the virus at the site of injury.

The Potency of Fowl-pox and Vaccinia.

Hens (Table I).—The virulence of the viruses of fowl-pox and vaccinia was tested by rubbing 0·25 c.c. of a given dilution of the virus into an area on the side of the head from which the feathers had been plucked. Strains A and B were active in dilutions of 1 in 100,000. Strain C, originally derived from the disease in a pigeon, could be transferred from the pigeon and passaged in the fowl. On first inoculating chicks with material from the pigeon the virus was found to be potent in dilutions up to 1 in 1000, but after repeated passages in the fowl it was found to be active in a dilution of 1 in 100,000, thus equalling the potency of strains A and B.

All races of hens were found to be equally susceptible to the three strains, there being no such racial specificity as is noted with the virus of the Rous sarcoma, which shows a predilection for birds of the Plymouth Rock variety. When using high dilutions of the virus of fowl-pox it is not uncommon to find some variation not only in the susceptibility of different birds but even of different areas of skin of the same bird.

Table I.—The Potency of Fowl-pox and Vaccinia Viruses for the Hen.

	Dilutions.				
	1 in 10.	1 in 100.	1 in 1000.	1 in 10,000.	1 in 100,000.
Fowl-pox A	+	+	+	+	+
,, B	+	+	+	+	+
,, C (on first passage from pigeon)	+	+	+	−	−
(After frequent passage in hen)	+	+	+	+	+
Vaccinia	+	+	−	−	−

+ = reaction positive. − = reaction negative.

Vaccinia virus, as contained in glycerinated calf lymph, produces in the skin of the chick only small yellowish papules which, though becoming pustular, generally remain discrete. Passage is only obtained with difficulty. If, however, the vaccinia virus is passaged to the chicken from lesions on the cornea of the rabbit or rat, a more vigorous reaction is obtained. A raised yellowish growth then appears, which macroscopically is indistinguishable from the lesion produced by fowl-pox. Even after more than 20 passages have been made in the chicken with vaccinia virus it still retains its full potency for the skin and cornea of the rabbit and the cornea of the rat. The virus of vaccinia is only active for the skin of the chick in dilutions up to 1 in 100.

Pigeons (Table II).—Fowl-pox strains A, B and vaccinia produced no lesions in the cells of the epidermis though there was always a slight reticulo-endothelial reaction in the dermis. No reaction followed the intravenous injection of these viruses into the pigeon, nor did digestion of the suspension of the viruses with trypsin render them virulent for the pigeon. Strain C, however, was active in dilutions up to 1 in 100,000. After transfer to the fowl and subsequent passage in this bird there was found to be no loss of potency for the pigeon after 10 passages, but after 30 passages the virulence for the pigeon had fallen to 1 in 1000. Further transference in the chick, up to 72 passages, did not reduce the potency for the pigeon, while full infectivity for the pigeon was quickly regained after one or two passages in this bird.

Table II.—Potency of Fowl-pox and Vaccinia Virus for the Pigeon.

	Dilutions.				
	1 in 10.	1 in 100.	1 in 1000.	1 in 10,000.	1 in 100,000.
Fowl-pox A	—	—	—	—	—
,, B	—	—	—	—	—
,, C	+	+	+	+	+
(After 30 passages in the hen)	+	+	+	—	—
Vaccinia	—	—	—	—	—

+ = reaction positive. — = reaction negative.

Ducks.—Numerous attempts were made to infect young ducklings with the viruses of fowl-pox and vaccinia but without a single positive result.

Rabbits (Table III).—Fowl-pox strains A, B and C injected intradermally into the rabbit or rubbed on a scarified area of skin produced slight reddening which lasted for from 2 to 3 days. When, however, the skin at the site of inoculation was removed 5 days after injection and rubbed on the skin of the chick positive results were obtained in 2 out of 8 experiments, thus showing that the virus of fowl-pox could at least retain its activity in the skin of the rabbit for some considerable period. All attempts to passage the virus in the skin of the rabbit were unsuccessful though the experiment was carried out at different times with 22 rabbits.

Vaccinia virus was potent for the skin of the rabbit in a dilution of 1 in 100,000. No diminution in virulence occurred after passage on the skin of the chick or cornea of the rat.

Table III.—Potency of Fowl-pox and Vaccinia Virus for the Rabbit.

	Dilutions.				
	1 in 10.	1 in 100.	1 in 1000.	1 in 10,000.	1 in 100,000.
Fowl-pox A	(+)	(+)	—	—	—
,, B	(+)	(+)	—	—	—
,, C	(+)	(+)	—	—	—
Vaccinia	+	+	+	+	+

+ = positive reaction. (+) = slight reaction. — = negative result.

Rats.—Ewing (1904) having shown that the cornea of the rat was very susceptible to inoculation with vaccinia, a constant supply of raw virus was obtained by passage on this animal's cornea. Vaccinia virus was constantly virulent in dilutions of 1 in 10,000, while occasionally a few rats showed a reaction with a dilution of 1 in 100,000. No reaction was obtained with any of the three strains of fowl-pox virus.

The Development of Natural Immunity to Fowl-pox.

It has long been recognised that birds which have recovered from a severe attack of fowl-pox are subsequently immune for a considerable period. Burnet (1906) attempted to determine the time of onset of immunity by reinoculating pigeons at variable intervals after a primary inoculation. A pigeon reinoculated on the fifth day had an eruption equivalent to that of the controls, but one reinoculated on the eighth day had the course of the disease considerably shortened, while by the twentieth day there was complete immunity. The experiments, however, were not of a quantitative character.

In the case of vaccinia in the rabbit Henseval and Convent (1910) found that by inoculating a fresh area of skin daily a certain degree of immunity had developed by the fourth day. Gordon (1925) has also found that by the fourth day the skin of the rabbit is partially immune to vaccinia, while by the eighth day this immunity is complete when the immunising virus is injected subcutaneously.

In order to determine the time at which immunity begins to appear in fowl-pox 16 chickens were rubbed with fowl-pox virus A on a wide area of skin at the back of the head and neck, from which the feathers had been removed. On the fourth, eighth, sixteenth and twentieth days four of the chicks were reinoculated on the skin of the side of the head with dilutions of the virus 1 in 100, 1 in 1000, 1 in 10,000, 1 in 100,000. The results (Table IV) show that by the fourth day there is a certain degree of immunity, which is complete by the twentieth day. Cross immunity tests carried out in chicks with the three strains A, B and C (Table V) prove that in the chick each strain is capable of immunising against itself and against the other two strains.

Table IV.—Development of Immunity against Reinoculation with Fowl-pox Virus.

Hens inoculated with strain A; subsequently reinoculated with dilutions of strain A.

No. of days between inoculations.	Dilutions.			
	1 in 100.	1 in 1000.	1 in 10,000.	1 in 100,000.
4	+	+	+	—
8	+	(+)	—	—
16	(+)	—	—	—
20	—	—	—	—

+ = fowl-pox produced. (+) = slight reaction. — = negative result.

Table V.—Development of Immunity against Reinoculation with Fowl-pox Virus.

(*a*) Hens inoculated with strain A; subsequently inoculated with strain B.

No. of days between inoculations.	Dilutions.			
	1 in 100.	1 in 1000.	1 in 10,000.	1 in 100,000.
4	+	+	(+)	—
8	+	(+)	(+)	—
16	+	(+)	—	—
20	—	—	—	—

+ = fowl-pox produced. (+) = slight reaction. — = negative result.

Table V—(continued).

(*b*) Hens inoculated with strain A; subsequently inoculated with strain C.

No. of days between inoculations.	Dilutions.			
	1 in 100.	1 in 1000.	1 in 10,000.	1 in 100,000.
4	+	+	(+)	—
8	+	+	—	—
16	+	(+)	—	—
20	—	—	—	—

+ = fowl-pox produced. (+) = slight reaction. — = negative result.

Similar results were obtained when strains B or C were used for the primary inoculation, immunity against both the homologous strain and the other two strains also being completely established by the twentieth day.

In the case of the pigeon inoculated with strain C immunity against the same strain was apparent by the fourth day and was complete by the twentieth day.

An attempt was made to determine whether any immunisation against strain C resulted in the pigeon from previous treatment with strains A or B. Five pigeons were scarified over a wide area of skin on the breast with strain A—no reaction characteristic of fowl-pox developed. At the same time they received intravenously 0·5 c.c. of a 1 in 10 dilution of strain B. Two subsequent injections of 1 c.c. of the same dilution of strain A were given on the tenth and twentieth days. On the latter day the five pigeons were inoculated by scarification with falling dilutions of strain C—1 in 10 to 1 in 100,000 (Table VI). The control pigeons were all positive, but of the prepared pigeons those inoculated with dilutions of 1 in 10,000 and 1 in 100,000 were negative, thus showing that previous treatment with strains A and B was capable of producing a slight degree of immunity to strain C in the pigeon, whereas in the chick strains A and B are capable of completely immunising against strain C.

Table VI.—The Potency of Strain C for Pigeons Immunised with Strains A and B.

Dilutions of strain C.	Controls.	Immune pigeons.
1 in 10	+	+
1 in 100	+	+
1 in 1000	+	+
1 in 10,000	+	—
1 in 100,000	+	—

+ = fowl-pox produced. — = negative result.

In order to test whether vaccinia virus could immunise against fowl-pox 15 chicks were inoculated over a large area of skin on the breast with chick passage vaccinia virus. Twenty days later they were reinoculated on the skin with vaccinia virus and were all found to be completely immune to vaccinial infection. They were then inoculated on the skin with dilutions 1 in 10 to 1 in 100,000 of fowl-pox virus strains A, B and C. All the chicks were positive, as were 15 control chickens inoculated with the same dilutions of strains A, B and C (Table VII).

Table VII.—The Potency of Strains A, B and C for Chickens Immunised with Vaccinia Virus.

Dilutions of virus.	Strain A.		Strain B.		Strain C.	
	Control.	Immune.	Control.	Immune.	Control.	Immune.
1 in 10	+	+	+	+	+	+
1 in 100	+	+	+	+	+	+
1 in 1000	+	+	+	+	+	+
1 in 10,000	+	+	+	+	+	+
1 in 100,000	+	+	+	+	+	+

+ = fowl-pox produced.

The reverse experiment was also performed. Five chicks were rubbed on the scarified skin with strain A. Twenty days later they were again rubbed with strain C and were found to be immune. Five days later they were rubbed with dilutions of chick passage vaccinia virus. All the chicks showed a positive reaction. There is thus no evidence from *in vivo* experiments to show that there is any cross immunity between the viruses of vaccinia and the three strains of fowl-pox employed.

The Duration of Immunity to Fowl-pox Virus.

A few experiments were carried out to determine in fowls and pigeons the duration of immunity to fowl-pox. Fifteen chicks and 15 pigeons were scarified over a wide area of skin on the head and neck, the chickens with strain A, the pigeons with strain C. Twenty days later the birds were all reinoculated with the same viruses and found to be immune. Fifty, 100 and 150 days later the birds were again inoculated with fowl-pox virus A. The results are shown in Table VIII.

Table VIII.

Dilution of virus A.	Chickens. Intervals after primary inoculation.					
	50 days.		100 days.		150 days.	
	Control.	Prepared chick.	Control.	Prepared chick.	Control.	Prepared chick.
1 in 10	+	—	+	(+)	+	+
1 in 100	+	—	+	—	+	+
1 in 1000	+	—	+	—	+	(+)
1 in 10,000	+	—	+	—	+	—
1 in 100,000	+	—	+	—	+	—

+ = fowl-pox produced. (+) = slight reaction. — = negative result.

Similar results obtained with pigeons and strain C show that 150 days after inoculation a slight degree of immunity still persists.

The Serology of Fowl-pox.

Although a considerable amount of work has been carried out on the serology of vaccinia in the rabbit, little attention seems to have been paid to the possibility of similar serological reactions occurring in birds suffering from fowl-pox. Burnet (1906) found that the serum of a pigeon which had recovered from the disease and one which had been hyper-immunised had no viricidal power. In his experiments an attempt was made to immunise fowls by giving them subcutaneously at weekly intervals doses of 10–20 c.c. of a dense suspension of the virus. One and a half months after the last injection they were bled and the serum separated out. A dilution of the virus mixed with an equal volume of the serum of a normal pigeon and with equal volumes of fowl immune serum, both heated and unheated, was allowed to stand for 1 and 3 days—no details are given of the temperature. The reaction on applying the mixtures to the scarified skin was equal both in the control bird and in the one treated with immune serum. In a further experiment immune pigeon and hen sera were thrice inoculated into the veins of normal pigeons in doses of 1 c.c. at intervals of 4 days. Nine days after the first inoculation the birds were inoculated on the scarified skin, as were also control birds. In the treated birds the incubation period was retarded 3 or 4 days and the reaction was at first slighter than in the controls, though later it developed normally. Two pigeons received intravenously 2 c.c. of the serum of a highly immunised fowl. The skin was inoculated 1 and 24 hours later. The reaction in the treated birds was less than in controls.

Löwenthal (1906) claims to have produced a skin immunity by intravenous injection of the virus, while Lipschütz (1908) makes similar claims following subcutaneous injection. Manteufel (1910) found that the serum of hens which had recovered from a typical attack of fowl-pox and had also been injected subcutaneously with fowl-pox virus had no therapeutic effect when injected into hens suffering from fowl-pox. Scarification of the combs of hens and inoculation with fowl-pox and simultaneous subcutaneous injection of immune serum was followed by a typical reaction. Immune serum and concentrated fowl-pox virus allowed to stand in contact for 2 to 3 days and then inoculated on the scarified comb of hens gave rise to a typical reaction. Hens injected subcutaneously with a mixture of immune hen serum and fowl-pox virus did not show any general reaction, as did hens injected with normal hen serum and fowl-pox virus. Some 14 days later, however, the hens injected with immune serum were found to be susceptible to infection with fowl-pox virus applied to the scarified comb.

van Heelsbergen (1920) also claims to have produced immunity to fowl-pox by three intravenous injections of fowl-pox filtrate which had been passed through a Chamberland B filter. Three hens inoculated subsequently on the scarified skin gave no reaction, while in five others the skin lesions were slight and were cured after 10 days; the control hens were all positive. Löwenthal, Kadowaki and Kondo (1924) failed to find any viricidal antibodies in the sera of pigeons, hens or rabbits after previous inoculation with pigeon-pox virus.

In a later communication, however, Löwenthal (1925) claims to have transmitted pigeon-pox to rabbits. After a few passages pigeon-pox protected against vaccinia, while the sera of rabbits recovered from pigeon-pox were said to have a viricidal action on the virus. Andervont (1926) was unable to detect any viricidal antibody in hens which had recovered from fowl-pox.

Although evidence of a certain degree of passive immunity has been obtained no clear evidence has been brought forward that the serum from immune fowls has any viricidal action on the virus of fowl-pox. Löwenthal, Kadowaki and Kondo (1924) have, however, succeeded in producing viricidal antibodies against vaccinia in the hen, although the titre was not as high as in the rabbit.

Preliminary experiments were therefore made to determine whether the serum of fowls which had recovered from the disease had any viricidal action on fowl-pox virus. Three fowls which had recovered from the disease produced by strain A were bled and their sera obtained. Dilutions of virus A

1 in 100, 1 in 1000, 1 in 10,000 were placed in contact with equal amounts of saline, of normal hen serum and of the three immune sera, the dilution of the serum being thus 1 in 2, and incubated for 3 hours at 37° C. in the water bath. The mixtures were then inoculated on the scarified skin of chicken, with the following result:—

Table IX.—Viricidal Action of Anti-fowl-pox Serum from Hens.

Dilution of virus.	Normal serum.	Immune sera.			Saline.
		1.	2.	3.	
1 in 100	+	+	+	+	+
1 in 1000	+	(+)	+	+	+
1 in 10,000	+	—	+	+	+

+ = fowl-pox produced. (+) = slight reaction. — = negative result.

The serum of fowl No. 1 having shown some evidence of a viricidal action on the virus this bird was injected intravenously with a 1 in 10 dilution of virus A. It received five injections beginning with 1 c.c. and rising to 5 c.c. at intervals of from 7 to 10 days.

The fowl was then bled and its serum separated off. In the first experiment dilutions of the serum were tested for viricidal action on dilutions of strains A and B 1 in 500 by the method used by Gordon (1925), except that the serum was unheated and guinea-pig serum was not used as a source of complement.

Table X.—Viricidal Action of Anti-fowl-pox Serum A on Strains A and B.

(1) Strain A: dilution 1 in 500.

Dilutions of serum.	Normal serum.	Immune serum.
1 : 2	+	—
1 : 10	+	—
1 : 20	+	—
1 : 40	+	(+)
1 : 80	+	+

+ = fowl-pox produced. (+) = slight reaction. — = negative result.

Table X—(continued).

(2) Strain B: dilution 1 in 500.

Dilutions of serum.	Normal serum.	Immune serum.
1 : 2	+	—
1 : 10	+	—
1 : 20	+	—
1 : 40	+	+
1 : 80	+	+

The viricidal action of anti-fowl-pox serum A on strains A and B was thus similar. The viricidal action of the same serum was then tested on a dilution 1 in 500 of strain C derived from fowl material. When the incubated virus was tested out, on both the fowl and the pigeon, the results were of a striking character.

Table XI.—Viricidal Action of Anti-fowl-pox Serum A on Strain C (Fowl Material) Tested on Fowls and Pigeons.

Dilutions of serum.	Normal serum.		Immune serum.	
	Hen.	Pigeon.	Hen.	Pigeon.
1 : 2	+	+	—	+
1 : 10	+	+	—	+
1 : 20	+	+	—	+
1 : 40	+	+	+	+
1 : 80	+	+	+	+

+ = fowl-pox produced. — = negative result.

The action of the anti-fowl-pox serum A on strain C had thus been to destroy its activity for the fowl, but not for the pigeon. On re-transferring some of the virus growth from the pigeon (1 : 2 dilution of serum) to the hen it was found to be pathogenic for the hen only in dilutions of 1 in 10, thus showing that the hen pathogenic portion of strain C had been almost entirely removed by the viricidal serum. A similar experiment was then carried out with the same anti-serum on strain C derived from the pigeon, the incubated virus being again tested on fowls and pigeons.

Table XII.—Viricidal Action of Anti-fowl-pox Serum A on Strain C (Pigeon Material) Tested on Fowls and Pigeons.

Dilutions of serum.	Normal serum.		Immune serum.	
	Hen.	Pigeon.	Hen.	Pigeon.
1 : 2	+	+	—	+
1 : 10	+	+	—	+
1 : 20	+	+	(+)	+
1 : 40	+	+	+	+
1 : 80	+	+	+	+

+ = fowl-pox produced. (+) = slight reaction. — = negative result.

Here again the potency of strain C for the fowl was destroyed while the potency for the pigeon remained. This is even more strikingly shown by testing the viricidal action of anti-fowl-pox serum A on dilutions of strain C derived from the fowl, the virus after incubation being tested out on both fowl and pigeon.

Table XIII.—Viricidal Action of Anti-fowl-pox Serum A (diluted 1 : 2) on Strain C (Fowl Material) Tested on Fowls and Pigeons.

Dilutions of virus C.	Normal serum.		Immune serum.		Saline.	
	Fowl.	Pigeon.	Fowl.	Pigeon.	Fowl.	Pigeon.
1 in 100	+	+	(+)	+	+	+
1 in 1000	+	+	—	+	+	+
1 in 10,000	+	+	—	+	+	+
1 in 100,000	+	+	—	(+)	+	+

+ = fowl-pox produced. (+) = slight reaction. — = negative result.

In view of the results obtained with anti-fowl-pox serum A it became essential to produce an anti-serum to strain C. Six fowls were immunised by producing the disease by scarification on the skin, followed by intravenous injection of dilutions of strain C obtained from the fowl.

Preliminary experiments showed that the sera of two out of the six fowls had a viricidal action on strain C. Serum was obtained from these two fowls and its viricidal action tested as before on strains A and B.

Table XIV.—Viricidal Action of Anti-fowl-pox Serum C on Strains A and B.

(1) Strain A: dilution 1 in 500.

Dilutions of serum.	Normal serum.	Immune serum.
1 : 2	+	—
1 : 10	+	—
1 : 20	+	—
1 : 40	+	+
1 : 80	+	+

(2) Strain B: dilution 1 in 500.

Dilutions of serum.	Normal serum.	Immune serum.
1 : 2	+	—
1 : 10	+	—
1 : 20	+	—
1 : 40	+	+
1 : 80	+	+

+ = fowl-pox produced. — = negative result.

The viricidal action of anti-fowl-pox serum C was then tested on strain C derived from the fowl and the pigeon, the incubated virus being inoculated both into fowls and pigeons.

Table XV.—Viricidal Action of Anti-fowl-pox Serum C on Strain C Tested on Fowls and Pigeons.

(1) Strain C (fowl material): dilution 1 in 500.

Dilutions of serum.	Normal serum.		Immune serum.	
	Hen.	Pigeon.	Hen.	Pigeon.
1 : 2	+	+	—	—
1 : 10	+	+	—	—
1 : 20	+	+	—	—
1 : 40	+	+	+	+
1 : 80	+	+	+	+

Table XV—(continued).

(2) Strain C (pigeon material): dilution 1 in 500.

Dilutions of serum.	Heated sera with complement.			
	Hen.	Pigeon.	Hen.	Pigeon.
1 : 2	+	+	—	—
1 : 10	+	+	—	—
1 : 20	+	+	(+)	(+)
1 : 40	+	+	+	+
1 : 80	+	+	+	+

+ = fowl-pox produced. (+) = slight reaction. — = negative result.

From these experiments it is seen that anti-fowl-pox serum C has the power of destroying the pathogenicity of virus C both for the pigeon and the fowl, while anti-fowl-pox serum A only destroys the pathogenicity for the fowl.

This strongly suggests that fowl-pox strain C is composed of two types of virus which are antigenetically distinct (1) a hen type; (2) a hen-pigeon type.

If one regards all the three strains A, B, C as having originated in the hen then C appears to contain certain mutant forms which are pathogenic for the pigeon, this difference in pathogenicity being correlated with differences both in immunising power and in serological reactions.

The Relationship between the Viruses of Fowl-pox and Vaccinia.

The view originally held by Jenner (1798) that the virus of vaccinia is a form of variola modified in its virulence for man by passage through the cow is now universally upheld. The relationship between vaccinia virus and the viruses producing stomatitis of the horse, sheep-pox and fowl-pox remains obscure.

Earlier observers such as Bollinger (1873) assumed without much evidence to support them, that fowl-pox was identical with vaccinia, relying possibly on the popular superstition of the middle ages that epidemics of fowl-pox preceded epidemics of small-pox in man. Later observers basing their views on the characteristic cell inclusions of fowl-pox and its relationship to fowl diphtheria came to regard vaccinia and fowl-pox as two distinct diseases. Reischauer (1906) however still maintained the older view on the ground that the inclusions characteristic of fowl-pox, despite their microchemical reactions, were identical with Guarnieri bodies as seen in variola and vaccinia.

Of more recent workers Zwick (1914) regards pustulous stomatitis of the horse and fowl-pox as closely related. The most convincing evidence, however, in favour of the identity of fowl-pox and vaccinia has been brought forward by Toyoda (1924). This observer found that fowl-pox scarified on the cornea or abdominal skin of the rabbit or guinea-pig gave a result which, both macro- and microscopically, was similar to that obtained with vaccinia. A lamb scarified with fowl-pox was subsequently found to be immune both to sheep-pox and vaccinia. Dogs first inoculated with sheep-pox were immune to vaccinia and fowl-pox. A child vaccinated with sheep-pox one month later gave no reaction with vaccinia or sheep-pox, while another child vaccinated with fowl-pox was immune to vaccinia.

van Heelsbergen (1920) in one experiment found that primary inoculation of the fowl with vaccinia gave rise to some degree of immunity against fowl-pox. In another experiment he was unable to demonstrate any such immunity.

van Nederveen (1926) also claims to have converted fowl-pox virus into vaccinia. A strain of fowl-pox virus was obtained from a hen which had the lesions on the feathered portions only. The first attempts to transmit the infection to the skin of rabbits were unsuccessful, but in a further experiment one rabbit showed a non-specific reaction on the skin. Material obtained from this lesion was transferred to other rabbits, and after the fourth passage both the skin and cornea gave a reaction similar to that obtained with vaccinia virus. Some of the material from the rabbit was then applied to the skin of a calf which gave a very definite reaction. From the calf the virus was transmitted to children with positive results, and back again to hens which showed lesions on the comb and, following intravenous injection, on the mucous membrane of the mouth.

Pandit (1927) was unable to transmit fowl-pox directly to the rabbit, but after passage in calves the virus became virulent for monkeys and rabbits. Calves which had been scarified with fowl-pox were subsequently vaccinated with calf lymph. At first the fowl-pox virus did not seem to immunise against vaccinia, but after passage in the calf distinct protection against vaccinia was afforded. Although precautions are said to have been taken against the possibility of confusing the viruses, it is noteworthy that these experiments were carried out in an establishment for the production of calf lymph.

On the other hand Levaditi, Nicolau and Harvier (1922) were unable to demonstrate any cross immunity in fowls between vaccinia and fowl-pox. Löwenthal, Kadowaki and Kondo (1924) by vaccinating chicks with vaccinia were able to produce a serum containing viricidal antibodies against vaccinia, but not against fowl-pox, while viricidal anti-vaccinia serum from the rabbit was also without action against fowl-pox. More recently Lusena (1925), Ledingham (1924) and Andervont (1926) have all failed to determine any cross immunity between fowl-pox and vaccinia.

It will be noted that among those who have claimed to transform the virus of fowl-pox into that of vaccinia there is considerable variation in the virulence for various animals of the strain of fowl-pox employed. Thus Toyoda was apparently able to transmit fowl-pox quite easily to the rabbit, guinea-pig or child ; van Nederveen only succeeded in transmitting the virus to the rabbit with some difficulty, while Pandit only infected the rabbit after passage through the calf. It is unfortunate that none of these observers carried out experiments to determine whether the virus of fowl-pox, when passaged on the rabbit or calf, still retained its power of traversing a Berkefeld or Chamberland filter. Nor, so far as we have been able to ascertain, has any attempt ever been made to convert the virus of vaccinia into that of fowl-pox by repeated passage in the chick, although such passage is easily carried out.

With the three strains of fowl-pox which we have employed it has not been possible to passage the infection in rabbits, nor has it been possible to produce by passage in the chick a form of vaccinia virus which lost its pathogenicity for the rabbit or rat.

Further attempts were made to transform the virus of fowl-pox into the virus of vaccinia by transmitting fowl-pox to calves. These experiments were carried out at the Stroud Laboratories, Mill Hill, where there were no animals infected with vaccinia. The calves were inoculated by rubbing the infected material on the scarified skin of the abdomen which had previously been shaved. Repeated attempts were made with strains A, B and C, but all gave negative results. Subsequent inoculation of the calves with vaccinia gave a typical positive reaction.

The experimental results which have previously been described show that there is no cross immunity engendered *in vivo*, but in order to determine more accurately if there were any antigenetic relationship between the virus of fowl-pox and that of vaccinia the following tests were carried out :—

(1) The viricidal action of an anti-vaccinia rabbit serum on fowl-pox viruses A, B and C.

(2) The viricidal action of an anti-fowl-pox A serum on vaccinia.

(3) The viricidal action of an anti-fowl vaccinia serum on vaccinia and fowl-pox.

The results are shown in the three following tables, the technique being similar to that used for demonstrating the viricidal action of anti-fowl-pox serum.

Table XVI.—Viricidal Action of Rabbit Anti-vaccinia Serum on Vaccinia (Tested in the Rabbit) and Fowl-pox A and C (Tested in the Fowl).

Vaccinia : dilution 1 in 200. Fowl-pox A and C : dilution 1 in 500.

Dilutions of serum.	Vaccinia.		Fowl-pox A.		Fowl-pox C.	
	Heated sera with complement from guinea-pig.					
	Normal.	Immune.	Normal.	Immune.	Normal.	Immune.
1 : 2	+	—	+	+	+	+
1 : 10	+	—	+	+	+	+
1 : 20	+	—	+	+	+	+
1 : 40	+	—	+	+	+	+
1 : 80	+	—	+	+	+	+

Controls with.	Vaccinia.	Fowl-pox A.	Fowl-pox C.
Heated normal serum	+	+	+
Heated immune serum	+	+	+
Complement	+	+	+
Saline	+	+	+

Table XVII.—Viricidal Action of Anti-fowl-pox A Serum on Vaccinia (Tested in the Rabbit) and on Fowl-pox A (Tested on the Fowl).

Vaccinia : dilution 1 in 200. Fowl-pox A : dilution 1 in 500.

Dilution of serum.	Vaccinia.		Fowl-pox A.	
	Normal.	Immune.	Normal.	Immune.
1 : 2	+	+	+	—
1 : 10	+	+	+	—
1 : 20	+	+	+	—
1 : 40	+	+	+	—
1 : 80	+	+	+	+

Table XVIII.—Viricidal Action of Fowl-anti-vaccinia Serum on Vaccinia and Fowl-pox A Tested in the Hen.

Vaccinia : dilution 1 in 50. Fowl-pox A : dilution 1 in 500.

Dilution of serum.	Vaccinia.		Fowl-pox A.	
	Normal.	Immune.	Normal.	Immune.
1 : 2	+	−	+	+
1 : 10	+	−	+	+
1 : 20	+	−	+	+
1 : 40	+	(+)	+	+
1 : 80	+	+	+	+

There is thus no evidence from serological observations that there is any antigenetic relationship between the viruses of fowl-pox and vaccinia.

The Filterability of Vaccinia and Fowl-pox Virus.

As is well known the factors which determine the filterability of ultra-microscopic organisms are extremely complex. Thus Levaditi and Nicolau (1923) believe that the main difficulty in filtering many viruses is due not to their size, but to the capacity for adsorbing these viruses possessed by substances such as kaolin, animal charcoal, and siliceous material such as that composing the Berkefeld filter. Other observers, among them Kramer (1926), regard the electric charge possessed by the filters and the organisms as the main factor in determining whether any given virus will or will not filter.

Many experiments have been carried out to determine the filterability of the vaccinia virus. Early observers such as Negri (1905 and 1906), Remlinger and Nouri (1905), Rouget (1905), Carini (1906), Nodake (1924) and Murata (1924), all claim to have obtained positive results with filtration through Berkefeld filters. Many of these observers fail to give any indication whether the filters were tested for their capacity to hold back visible pathogenic bacteria. Levaditi and Nicolau (1923) state that the virus of vaccinia passes more readily through a collodion membrane than the viruses of rabies, encephalitis or herpes. Thomson (1924) and many others, on the other hand, have entirely failed to obtain any evidence that the virus of vaccinia is filterable.

Twenty-four experiments were carried out by us on the filterability of the virus of vaccinia, the material being obtained either from the skin of the rabbit or the cornea of the rat. In four experiments the suspension of tissue pulp was first submitted to digestion with a solution of trypsin for 5 hours at 38° C., as recommended by Nicolle and Adil Bey (1906). The filters used in each experiment were Berkefeld V and Chamberland L_1. The suspension of the virus freed from cells by slow centrifugation was first passed through the filter candle, after which a suspension of a streptococcus was filtered to determine the capacity of the candle to hold back bacteria. The filtrate from the suspension of the virus was inoculated on to the rabbit's skin and rat's cornea, but in no experiment was there any evidence that any vaccinia virus had passed through the candle.

The filterability of the virus of fowl-pox was first demonstrated by Marx and Sticker (1902) and that this virus is easily filterable is shown by the following experiments :—Dilutions in saline of fowl-pox virus strain A were prepared of 1 in 100, 1 in 1000, 1 in 10,000 and 1 in 100,000. The potency of these dilutions was then tested on the skin of the fowl, before and after filtration through Berkefeld filters. While the unfiltered material gave a positive result in a dilution of 1 in 100,000, the filtered material was positive in a dilution of 1 in 10,000. Similar results were obtained in filtration experiments with strains B and C.

Since the virus of vaccinia produces exactly similar lesions in the skin of the chick to those caused by the virus of fowl-pox it seemed possible that the vaccinia virus from the lesions in the chick's skin might also be filterable.

Eighteen experiments were carried out with the vaccinia virus from the chick's skin, the same precautions being taken as before to control the filter. The *p*H of the suspension of vaccinia virus was the same as that of the suspension of fowl-pox virus. The filtrate was inoculated into the skin of the rabbit, and in one experiment out of the 18 a positive reaction was obtained. It must, however, be remembered that in testing the filter candles with a suspension of streptococcus, after the filtration of the virus suspension, some minute holes which had allowed the passage of the virus may be subsequently blocked by the virus suspension, thus rendering the candle apparently capable of holding back bacteria.

As the result of these experiments, however, it can be definitely said that the virus of vaccinia does not possess the same capacity for traversing Berkefeld or Chamberland L_1 candles as the virus of fowl-pox, even though the viruses are present in the same tissue substrate.

The evidence obtained from experiments on pathogenicity, immunity *in vivo*, viricidal action of immune sera, and filterability all goes to show that there is no direct relation between the virus of vaccinia and the three strains of fowl-pox virus which we have investigated. As our experiments have shown, however, there are differences between various strains of fowl-pox virus, and it is always possible that certain strains of fowl-pox may have some affinity with the virus of vaccinia.

Another possible explanation for the results obtained by Toyoda, Pandit and van Nederveen is that the original hens from which these observers obtained their material were infected, not with the virus of fowl-pox, but with the virus of true vaccinia, a virus which, as cytological observations show, produces in the fowl lesions which cannot be distinguished microscopically from those caused by the fowl-pox virus.

Conclusions.

1. Three strains of fowl-pox virus have been studied: two of these are pathogenic for the fowl only, while one is pathogenic for the fowl and the pigeon.

2. All attempts to render these three strains pathogenic for rabbits, rats, calves and ducks have failed.

3. One attack of fowl-pox produces immunity. In the fowl any one of the three strains completely immunises against the others, but in the pigeon previous treatment with the strains pathogenic for the fowl produces only partial immunity against the strain pathogenic for the pigeon and fowl.

4. Hyperimmunisation with fowl-pox virus produces in the fowl an anti-serum containing viricidal antibodies.

5. An anti-serum prepared against one of the fowl pathogenic strains destroys the pathogenicity of both the viruses which are pathogenic for the fowl only, but when acting on the virus pathogenic both for fowl and pigeon destroys only its pathogenicity for the fowl.

6. Vaccinia virus passaged in the fowl does not become converted into fowl-pox virus and remains pathogenic for the rabbit and rat.

7. Vaccinia virus and fowl-pox virus do not show any cross immunity in the hen.

8. An anti-vaccinia serum from the hen or rabbit has no viricidal action on the virus of fowl-pox, nor has an anti-fowl-pox serum from the hen any viricidal action on the virus of vaccinia.

9. While fowl-pox virus easily passes through a Berkefeld filter, vaccinia virus even after repeated passage in the hen is still largely held back.

10. No evidence has been obtained to show any close relationship between the virus of vaccinia and the three strains of fowl-pox virus investigated.

11. The factors underlying the Calmette-Guérin phenomenon are discussed

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The Air-Resistance to a Runner.

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(Received December 15, 1927.)

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The resistance of the air to the motion of a badly stream-lined body, in which the negative pressure on the leeward side, due to eddy formation, is appreciable, may be roughly $0 \cdot 6\ \rho v^2 A$, where v is the relative velocity, ρ the density of the air, and A the projected area on a plane perpendicular to the direction of motion. In a recent paper Furusawa, Hill and Parkinson (1) have examined the motion of a runner making a maximal effort over a short distance, on the assumption that the air-resistance is negligible when compared with the resistances inherent in the muscles and limbs of the runner himself. This assumption is nearly, but not strictly, true, the air-resistance to a man running in still air at maximum speed being of the order of 2 to 5 per cent. of the total resistance which his muscles have to overcome.

Model of runner used in wind-channel experiments; spindle visible at top.

In view of the importance of fractions of a second in sprint "records," and of the well-known influence of a following or head-wind in diminishing or increasing the time in such races, the original treatment of the dynamics of sprint-running may be modified to take account of the air-resistance as follows.

In calculating the air-resistance at any given relative speed it is necessary to know (1) how accurately the above formula fits and (2) the value of A, the projected area of a running man. In this connection I am much indebted to Sir T. E. Stanton, F.R.S., Superintendent of the Engineering Department of the National Physical Laboratory, who arranged for experiments to be performed in a 3-foot wind-channel on the rough model of a running man, shown in the figure. To quote his report, " the model, which is a ' lay-figure ' 8 inches high in the erect position, was fixed to the balance spindle as shown, and the resistances at different wind-speeds determined. The measured forces were then corrected for spindle interference in the usual way. To determine the projected area of the model a photograph was taken and its area measured by a planimeter. The measured areas of the model were :—

In the running position, 9·36 square inches.
In the erect position, 10·97 square inches.

The resistance, which varied as the square of the speed, is given by the following expressions :—

$$R = 0{\cdot}45\ \rho v^2 A_r \text{ lbs. in running position} \qquad (1)$$

$$R = 0{\cdot}49\ \rho v^2 A_e \text{ lbs. in erect position} \qquad (2)$$

where

A_r = projected area in running position (square feet).
A_e = projected area in erect position (square feet).
v = speed in feet per second.
ρ = 0·00237."

In the above formulæ ρ is the density of the air at 15° C. and at normal barometric pressure, expressed in lbs. per cubic foot, and divided by g (32·2 f.s.s.) in order to reduce the resistance to lbs. weight.

In C.G.S. units, if v be the velocity in centimetres per second, A the area in square centimetres, and R the resistance in grams weight, the value of ρ would be $1{\cdot}247 \times 10^{-6}$. Expressing the velocity in metres per second, the area in square metres, and the resistance in kilograms weight, ρ would be 0·1247. Thus the most convenient formulæ for practical purposes are :—

(A) Velocity in feet per second, area in square feet, resistance in lbs.

$$R = 0{\cdot}00107\ v^2 A_r \text{ (running)}$$

$$R = 0{\cdot}00116\ v^2 A_e \text{ (erect)}$$

(B) Velocity in metres per second, area in square metres, resistance in kilograms.

$$R = 0{\cdot}056\ v^2 A_r \text{ (running)}$$

$$R = 0{\cdot}061\ v^2 A_e \text{ (erect)}$$

According to Sir T. E. Stanton's measurements the lay-figure, 8 inches high, had a projected area of 9·36 square inches (running) and of 10·97 square inches (erect), so that its area bore to the square of its height a ratio of 0·146 (running) and 0·171 (erect). Thus a man 5 feet 9 inches high should have a projected area of 4·8 square feet (running), and 5·6 square feet (erect), were he similar to the model. To verify this a photograph of a runner finishing a race was measured up, and its area found to be about 0·16 times the square of its height. Thus the projected area of a runner of ordinary size may be taken as approximately 5 square feet, and the resistance to his motion as about $0 \cdot 0053\, v^2$ lbs., where v is the relative speed in feet per second.

The greatest speed ever measured in a human runner in still air is (1, p. 49) about 11·5 yards, *i.e.*, 34·5 feet per second. At this speed the calculated resistance would be 6·3 lbs.; at 24 feet per second it would be only 3 lbs. This runner (1, p. 50) at top speed was exerting a propelling force of about 80 per cent. of his weight, viz., about 135 lbs. Since the maximum attainable speed is proportional to the net propelling force and inversely proportional to the coefficient of internal viscous resistance, it follows that if the air-resistance could be abolished the maximum speed would be increased nearly 5 per cent.; this would occur with a following wind of about 24 miles per hour, in which a first-rate runner should be able to improve on his time in 100 yards by about 0·5 second. Similarly, a following wind of 10 miles per hour should improve his time by about 3 per cent., *i.e.*, in 100 yards by about 0·3 second. Conversely a head-wind of 10 miles per hour should double the air-resistance and increase his time by 0·5 second in the same distance. It would be of interest to measure the maximum speed of an athlete experimentally by electrical methods, as described in (1), when running with and against a wind of known strength, and to compare the results with the preceding calculation.

The matter can be expressed in another rather instructive way, viz., by estimating the air-resistance in terms of an "equivalent slope," *i.e.*, in terms of a hill which would require the same effort to overcome. Assuming a subject of 160 lbs., an air-resistance of 8 lbs. would, for example, be equivalent to a hill of 1 in 20.

Relative velocity of air:

Feet per second	0	10	20	30	40	50	60	80
Equivalent slope	0	1/300	1/75	1/33	1/19	1/12	1/8	1/5

At high relative speeds it is seen that air-resistance becomes equivalent to a steep hill, as of course is known to those who have walked, or attempted to run, against a strong wind.

Races of different lengths are run at different speeds, and it is instructive to calculate the resistances (assuming still air, a projected area of 5 square feet and a subject of 160 lbs.) for the speeds at which various "world's records" are performed. The last column gives the horse-power required to overcome the air-resistance.

Distance.	Time.		Average speed.	Resistance.	Equivalent. slope.	Horse-power.
	min.	sec.	feet per sec.	lbs.		
100 yards	0	9·3	32·3	5·5	1/29	0·32
220 ,,	0	20·8	31·7	5·3	1/30	0·30
300 ,,	0	30·2	29·8	4·7	1/34	0·26
440 ,,	0	47·4	27·8	4·1	1/39	0·21
600 ,,	1	10·8	25·4	3·4	1/47	0·16
880 ,,	1	51·6	23·7	3·0	1/54	0·13
1000 ,,	2	12·2	22·9	2·8	1/58	0·12
1320 ,,	3	2·8	21·7	2·5	1/64	0·10
1 mile	4	10·4	21·1	2·4	1/68	0·09
2 ,,	9	9·6	19·2	2·0	1/82	0·07
3 ,,	14	11·2	18·6	1·85	1/87	0·06
5 ,,	24	6·2	18·3	1·8	1/90	0·06
10 ,,	50	40·6	17·4	1·6	1/100	0·05

In the longer races, neglecting altogether the large oxygen debt at the end, there will be an oxygen consumption at the rate of at least 4 litres per minute, in a subject of the size assumed. This, if used in the combustion of glycogen, would yield (2, p. 6) energy at the rate of 63,400 foot-lbs. per minute, *i.e.*, of about 1·92 h.p. Thus only a very small fraction of the total energy liberated (3 per cent.) is employed in overcoming air-resistance. In running at maximum speed a first-rate "sprinter" may be developing about 8½ h.p. (1, p. 50). To overcome the air-resistance requires only about 4 per cent. of this amount.

We see, therefore, that still air provides a small, but not quite negligible, fraction of the whole resistance to a runner. In the finest athletes at top speed this fraction may amount to nearly 5 per cent., in others it will be considerably less, since it varies as the square of the speed. Against a head-wind it may be considerably greater. During the acceleration at the beginning of a race it will be relatively unimportant, since the speed is lower: thus in very short races (*e.g.*, 30 to 50 yards) the wind will have little influence. In the mathematical treatment of the dynamics of a sprinter (1, p. 34) the air-resistance was neglected; it would not be difficult so to adjust the calculations as to take approximate account of it, but the result would have little added significance, since the correction at the low velocities near the start would be almost negligible. It is simpler therefore, and practically as accurate, to do as we have done, viz., to treat the problem first as though there were no air-resistance and then to apply the necessary correction to the constants so obtained.

It may be of interest to note, paradoxical as it may seem at first, that a strong cross-wind may be a considerable hindrance to a runner. Let v be the velocity of the runner, V that of the cross-wind, the resultant relative velocity of runner and air is $\sqrt{v^2 + V^2}$, and this makes an angle $\cos^{-1}\frac{v}{\sqrt{V^2 + v^2}}$ with his direction of motion. The air-resistance being proportional to the square of the relative velocity, the component opposing the runner is proportional to $(v^2 + V^2)\frac{v}{\sqrt{v^2 + V^2}}$, *i.e.*, to $v\sqrt{v^2 + V^2}$; in still air this reduces to v^2, so that a cross-wind increases the head resistance in the ratio $\sqrt{1+\frac{V^2}{v^2}}$. In the case of a wind of the same order of size as the speed of the runner this increase may be considerable. For example, if $V = v$ the air-resistance is increased 1·41 times. Thus apart altogether from the disturbance to gait there is a considerable addition to the head resistance.

In running on a "circular" track it is obvious that a head-wind over part of the course is not compensated for by a following wind over the other part, since the resistance varies as the square, and not as the first power of the speed. This, together with the effect of a cross-wind over the remaining half of the track, explains the difficulty of doing good "times" on a windy day.

Summary.

1. The air-resistance to a model of a runner has been measured in a wind-channel, and found to be $0{\cdot}45\,\rho v^2 A$, where ρ is the density of the air, v the relative velocity, and A the projected area of the model in the direction of motion. In practical units the resistance is $0{\cdot}00107\,v^2 A$ lbs. weight, where v is in feet per second and A in square feet.

2. The projected area of a runner is approximately 0·15 times the square of his height.

3. From these data the air-resistance can be calculated. For a runner on a still day the air never provides more than 5 per cent. of the total resistance, the remainder being in his muscles and limbs themselves. A following wind of 10 miles per hour will increase the speed by about 3 per cent., a similar head-wind will diminish the speed by about 5 per cent., in a first-rate performer, in others less.

4. A table is given of the air-resistance experienced at various speeds; and the effect of a cross-wind is discussed.

5. It is shown that the correction to be applied for air-resistance is too small

to affect materially the equations previously found to govern the acceleration of a runner at the beginning of a race.

My sincere thanks are due to Sir T. E. Stanton, F.R.S., for calling my attention to the possibility of these calculations and for making the necessary observations in a wind-channel at the National Physical Laboratory.

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Changes in the Ovary of the Mouse following Exposure to X-Rays. Part IV.—The Corpus Luteum in the Sterilized Ovary, and some Concluding Experiments.

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(Communicated by Prof. J. P. Hill, F.R.S.—Received November 22, 1927.)

(From the Department of Anatomy, and the Department of Physiology and Biochemistry, University College, London.)

[Plate 23.]

CONTENTS.

I. Introduction 385
II. Irradiation of the adult during pregnancy 386
III. Irradiation of the adult during lactation 388
IV. Unilateral ovariotomy after sterilization at 3 weeks old 390
V. Irradiation of regenerate ovarian tissue 391
VI. Miscellaneous experiments 391
VII. Discussion 394
VIII. Summary 395

I.—*Introduction.*

The previous papers of this series dealt only in a brief and general manner with the effect of X-ray sterilization on the corpora lutea. The present paper, the last of the series, includes more extensive work aimed at removing this deficiency. The work was begun by irradiating pregnant animals, but owing to the technical difficulties the greater part of it was carried out on lactating animals. In this paper it will be shown that the degenerative changes in the corpora lutea of pregnancy and lactation take place very slowly after sterilization, although they begin at the normal time. The corpora lutea become, in fact, practically permanent structures. The œstrous cycles of both these series of animals are dealt with elsewhere (9) by one of us, where it is shown that these "permanent" corpora lutea do not retain their œstrous-inhibiting power for any appreciable period after what would be the functional life of the normal corpus luteum. Moreover, the possible duration of lactation is not affected by sterilization at the beginning of suckling.

The histology of the ovaries discussed in this paper is essentially similar to that found in the animals previously described (1, 2, and 3) and need not be dealt with at length.

The previous literature is summarised in the earlier papers (1, 2).

Our thanks are again due to Prof. J. P. Hill, F.R.S., Dr. F. H. A. Marshall, F.R.S., and Dr. J. A. Murray, F.R.S., for advice and criticism, while we are indebted to Prof. C. Lovett Evans, F.R.S., for his interest in the work. We also wish to thank Prof. G. Elliot Smith, F.R.S., for allowing the irradiation to be carried out in the Department of Anatomy.

The mice were drawn from the colony maintained with the aid of grants from the Government Grant Committee of the Royal Society and the Medical Research Council to A.S.P., and the histological expenses were defrayed from a grant to F.W.R.B. from the former source.

II.—*Irradiation of the Adult during Pregnancy.*

The abnormal persistence of corpora lutea (vera and atretica) in ovaries sterilized by X-rays, described previously (1, 3), suggested that sterilization of the pregnant adult might cause the persistent corpora lutea to function for an abnormally long period. Various doses were employed, as the animals concerned were mostly irradiated before a standard intensity had been worked out.

Twenty-eight animals, comprising three series (PX, SX and XVP) were irradiated in all. A list of these mice is given in Table I.

The ovaries of the XVP series were fixed in Bouin's fluid and stained with Ehrlich's hæmatoxylin and eosin, the results being good. The ovaries of the PX and SX series were fixed in Champy's or Carnoy's fluid and stained with Ehrlich's hæmatoxylin or Heidenhain's iron-hæmatoxylin.

Table I.—Mice Irradiated during Pregnancy.

Reference No.	Time when irradiated in days.	Dose (in minutes).	Time of autopsy in days after irradiation.	Sterility.	Time of autopsy in days after last œstrus.
XVP 11	5 p.c.	40	1	—	—
,, 12	5 ,,	40	3	—	—
,, 13	5 ,,	40	6	—	—
,, 1	6 ,,	40	72	+	27
,, 2	5 ,,	40	72	+	9
,, 3	5 ,,	40	72	+	35
,, 4	4 ,,	40	72	—	17
,, 5	4 ,,	40	72	+	24
,, 9	5 ,,	40	102	+	55
,, 7	5 ,,	40	109	+	(cornified)
PX 10	18 p.c.	40	21	—	—
,, 4	14 ,,	10	28	—	—
,, 7	16 ,,	40	28	—	—
,, 5	16 ,,	40	42	+(?)	—
,, 6	18 ,,	40	84	+(?)	—
,, 3	18 ,,	10	91	—	—
,, 2	14 ,,	10	137	—	—
,, 1	9 ,,	10	140	—	—
SX 2	14 p.c.–2 p.p.	30	61	+(?)	—
,, 4	17 ,, –5 ,,	30	93	+(?)	—
,, 5	15 ,, –3 ,,	30	93	+(?)	—
,, 1	15 ,, –3 ,,	30	105	+(?)	—

80 minutes' exposure was approximately equal to ½ B tint on Levi's pastilles.

The animals XVP 11, 12 and 13 afford earlier stages after irradiation than the PC series to be described in the next section. They are all devoid of small oocytes in primordial or single-layered follicles, which must therefore have degenerated within 24 hours of irradiation. Some small anovular follicles have been formed in all three animals by the rapid degeneration of the oocytes in some of the smaller follicles. Many of the larger follicles also show signs of degeneration.

None of the animals described in this section show any signs of proliferation and ingrowth from the germinal epithelium, such as has been described in the animals irradiated before puberty. The tissues of the sterilized ovary are derived from the interfollicular elements and the derivatives of the disorganised follicles in a similar manner to that described previously in the adult (3). Corpora lutea are present in the sterilized ovaries in all cases and invariably show more or less advanced signs of degeneration, such as fibrosis, shrinkage and vacuolation of the luteal cells.

It is thus clear that degeneration of the corpora lutea is very slow in the sterilized animal. All the sterilized ovaries contain some anovular follicles. It is of especial interest that XVP 1-5 and 9 all showed a more or less prolonged diœstrus before killing, and all had a remarkably luteal-like development of the interfollicular tissue (Plate 23, fig. 3). Further, in XVP 7, which was persistently cornified prior to being killed, the interfollicular tissue is markedly vacuolated (Plate 23, fig. 2). A similar condition and correlation has been noticed in some of the irradiated animals already described. In other respects the fuller descriptions of the non-parous adults (3) and of the PC series (in the next section) are applicable to these animals and need not be repeated here.

III.—*Irradiation of the Adult during Lactation.*

In view of the difficulties presented by irradiation during pregnancy (see Parkes (9)) it became clear that sterilization during lactation would probably afford more valuable and uniform results.

Forty-two lactating mice were irradiated for the purposes of this experiment. The first five were given a dose equal to ⅛ B tint on Levi's pastilles. These animals had been mated at the post-partum œstrus and all were pregnant and lactating when irradiated. The ovaries of only two of these animals were subsequently found to have been completely sterilized. The next 22 animals were given double the dose (= ¼ B tint Levi's pastilles). Three others were killed under 30 days after irradiation, and before the sterilization changes could be expected to be complete. Eleven of the remaining 19 were found to be completely sterilized. The last 15 animals were given a dose equivalent to ½ B tint on Levi's pastilles, and 12 of these were found to be completely sterilized. Twenty-five of the 42 animals were thus sterilized.

Further details of the histories of these animals, the method of irradiation and of the detection of œstrus are given in a separate paper by one of us (9).

The ovaries in the majority of cases were fixed in Bouin's fluid and were stained with Ehrlich's hæmatoxylin and eosin, Heidenhain's iron-hæmatoxylin, or Pasini's stain. In some cases Carnoy's or Regaud's fluid was employed, but neither gave very satisfactory results.

After the first post-partum œstrus the normal lactating mouse does not again come into œstrus for, on an average, about 25 days (6). This œstrus inhibition during lactation is almost certainly produced by the corpora lutea lactationes (7). Subsequent to this œstrous period the cycle is similar to that of the non-pregnant and non-lactating adult. Lactation can be prolonged to at least 90 days in some cases by supplying the female with successive foster-litters, when each is ready to be weaned. This prolonged lactation does not, however, result in a lengthening of the initial long diœstrous period or of the subsequent œstrous cycles. It has been shown (9) that the "permanent" corpora lutea in the sterilized ovaries of the animals described in this paper did not produce any significant deviation from the normal length of the initial diœstrous period. Further, the possible duration of lactation was not effected by sterilization.

The histological changes in the ovaries of the present series of animals following irradiation were practically identical with those observed in the non-parous adults (3), and need only brief description here. The absence of post-irradiation proliferation of the germinal epithelium, which remained thin and inactive, was equally clear in all animals. The tunica albuginea was intact and well developed in every case. The conclusion, that the tissues of the sterilized ovaries were derived entirely from the elements already composing the cortex at the time of irradiation, is therefore inevitable and confirms that drawn from the non-parous adults. The degeneration of oocytes and follicles took place in a similar manner to that previously described (3). The small oocytes disappeared with extreme rapidity. None of the animals killed under 42 days after irradiation were completely sterilized. Anovular follicles were formed from some of the smaller follicles by degeneration of the contained oocytes. They were present, in small numbers, in most of the ovaries.

The formation of corpora lutea atretica was observed in a few cases (Plate 23, fig. 1), but might have been overlooked in others on account of being almost indistinguishable from corpora lutea vera, which were present in large numbers. The tissues of the sterilized ovaries were very luteal-like in some of the animals that were killed a long time after irradiation, as was the case in some of the animals X-rayed before puberty. Most of the cases with this luteal-like development exhibited a stoppage of the œstrous cycle (*i.e.*, PC 6, 7, 10, 12, 13 and 14). This tissue was derived from the hypertrophied interfollicular tissue and from follicular elements which appeared to survive in the ovaries after the follicles as such had disappeared. The medulla remained practically unchanged. The ovaries varied somewhat in size but were, on the whole, not abnormally small. In these respects therefore the present series of animals entirely confirms the results obtained with the non-parous adults, and the conclusion that the different behaviour of the latter as compared with the animals X-rayed before puberty was a characteristic of the adult condition.

It remains to describe in somewhat more detail the condition of the corpora lutea, as previously this problem was intentionally left over for the present paper, where its discussion is more appropriate. Corpora lutea are present in all except two of the sterilized animals. The tissues of the ovaries of these two, PC 12 and 13, have become very luteal-like in character, which may account for the failure to distinguish any definite corpora lutea in them. All the corpora lutea in the sterilized ovaries exhibit obvious signs of retrogression, such as shrinkage of the entire body and of the luteal cells, vacuolation and fusion of the latter, leucocyte infiltration and fibrosis. In many of the animals two or three distinct generations of corpora lutea can be distinguished in various stages of degeneration. The most degenerate corpora lutea are represented by small fibrosed, rather indefinite, masses in the ovary, each containing a few scattered lutein cells, very old and vacuolated. The corpora lutea in the animals kept longest after irradiation are mostly in this condition. It is only possible in a few of the earlier cases to distinguish the corpora lutea of pregnancy and lactation from those of ovulation. In the other cases degeneration has proceeded too far for this distinction to be made.

In one animal, PC 14, a number of remarkable multi-nucleated giant cells, isolated or in groups, are present in the ovaries (Plate 23, fig. 4). These giant cells have a vacuolated cytoplasm and as many as 20 apparently healthy nuclei. They are separated from each other by thin connective tissue trabeculæ. They appear to be formed by a degenerative fusion on a large scale of the lutein cells in the old corpora lutea, and possibly of some of the luteal-like cells composing the remainder of the tissue of the ovaries.

IV.—*Unilateral Ovariotomy after Sterilization at 3 Weeks Old.*

Three of the series of animals irradiated at 3 weeks old (1) had one ovary removed after sterilization, and it seemed desirable to ascertain whether the remaining ovary underwent hypertrophy, as happens in the normal animal after unilateral ovariotomy.

The essential information is summarised in Table II.

Table II.

No. of animals.	Time of autopsy after removal of left ovary.	Mean diameter of left ovary.	Mean diameter of right ovary.	Percentage increase in diameter of right ovary over diameter of left ovary.
	Days.	μ.	μ.	Per cent.
M 14	34	1044	1055	+ 1·1
M 15	79	1077	1446	+34·3
M 16	79	1032	1664	+61·2

In the first case the increase, though positive, is insignificant. In the other two the increase is very great and can hardly be accounted for by the slight variation in size often experienced between the right and left ovaries. It therefore seems to represent a true compensatory hypertrophy of the sterilized ovary after unilateral ovariotomy, such as is well known after unilateral ovariotomy in the normal animal. It is possible that the increase in size in the first case would have been greater if it had been allowed to live as long after the operation as the other two, and might possibly then have been of an order comparable to that exhibited by them. It is more probable, however, that the failure definitely to hypertrophy in this case was associated with the very vacuolated luteal-like character of the tissues of the ovary and with the stoppage of the œstrous cycle.

V.—*Irradiation of Regenerate Ovarian Tissue.*

In another paper (8) the details have been given of the irradiation of two animals (OVS 17 and 23) suspected of having regenerated ovarian tissue after double ovariotomy. Subsequent dissection and microscopical examination of the ovarian region showed the presence of typical irradiated ovarian tissue. This tissue in both animals was embedded in and completely surrounded by the fat-body, as in the other cases of ovarian regeneration described. It consisted of cords of rather luteal-like cells separated by thin sheaths of connective tissue, such as have been described (1, 2) as forming the major part of the ovaries of animals irradiated before puberty. Corpora lutea, oocytes and follicles were entirely absent from this tissue, as were also definite "anovular" follicles. Several small tubules, similar to the persistent rete and mesonephric tubules found in the hilum of the normal ovary, were associated with this mass and appeared in places to merge and fuse with the cords of cells composing it. The whole ovarian mass in both cases was surrounded by a thin, more or less indefinite, fibrous connective-tissue sheath, which separated it from the adjoining adipose cells of the fat-body. It was in both cases situated near to the cystic remains of the old ligatured Fallopian tube, and the vascular supply was rich. These results indicate that regenerating ovarian tissue behaves similarly, after X-ray sterilization, to the ovaries of immature animals.

VI.—*Miscellaneous Experiments.*

The striking nature of the changes which take place in the ovary of the mouse after exposure to X-rays suggested that alteration of the conditions under which these changes normally take place in the irradiated animal might lead to alteration in the character of the changes themselves and possibly to fresh light being thrown on the effects of irradiation. In particular, it was desired to clear up, if possible, the actual nature of the anovular follicle-like structures found in practically all irradiated ovaries. In view of the essential similarity of these bodies with the ovarian structures described by Hammond (4) as spermatic tubules, and their similarity with the tubules found in the free-martin gonad, it was at first thought (1) that these structures found in the irradiated mouse gonad might also be of the nature of spermatic tubules. Subsequently, however, the fact that these structures could be produced in a day by irradiation of the adult, and were obviously of follicular derivation, made it necessary to suppose that, whether they had ever contained an ovum (as in irradiated adults) or not (as in animals irradiated before puberty) they were probably anovular follicles.

Nevertheless, certain of the experiments described in this paper were based on the possibility that these structures might be rudimentary spermatic tubules and were designed to facilitate their development as such. The development of testis elements in the ovary would presumably be retarded by the fact that their situation would be analogous to that found in the undescended testis, which is usually sterile, and might also be retarded by their female environment. With these considerations in view animals, both young and adult, were prepared in which the ovaries were retracted through the body wall and fixed under the skin, to give some semblance of the scrotal position.

This operation (left ovary only) was performed on three young mice (DM 1–3) at three weeks old, and 5 and 12 days later 40 minutes' exposure to X-rays, (totalling ½ B tint) was given. DM 3 showed poor growth and was killed a month later, but DM 1–2 were in good condition when put on to vaginal examinations at 8 weeks old. DM 2 showed a series of quite normal cycles, but DM 1 had an excessive amount of cornified material in the vagina during the whole time that examinations were made.

Autopsy of DM 1 and 2, made at 12 weeks old, showed that the left ovaries were satisfactorily fixed on the external surface of the body wall, but nothing of interest was found when the ovaries were examined histologically.

Three adult animals were also operated on in this manner, one after irradiation and two before. M 24 was given ½ B tint in two exposures at 3 weeks old, and when adult was examined for 40 days with no apparent signs of œstrus. This, therefore, appeared to be another case suitable for further experiment, and the right ovary was, therefore, retracted from the body cavity and fixed under the skin. Examination was continued for another 7 weeks, but still no definite signs of cyclic œstrous activity were observed. Autopsy was then performed, the animal being 21 weeks old, but microscopic examination again showed no features of interest.

NVS 12 was observed to have regular cycles. At the end of the seventh cycle (attended by copulation) the ovaries were retracted and fixed under the skin. The next period of œstrus (with copulation) occurred normally in 13 days. During the next cycle, 14 days in duration, ¼ B tint irradiation was given in two exposures, copulation occurring on the day following the second exposure. The next œstrous period occurred 24 days later, and was accompanied by copulation. In view of the very regular œstrous history of the animal, this long cycle suggests some disturbance in the œstrous-producing mechanism. Examination was continued for a further 133 days, but no further signs of œstrus were observed. The left ovary was removed 27 days after the last period of œstrus, and autopsy was performed 106 days later, 266 days after the beginning of daily vaginal examinations.

Histologically, the ovaries were found to be composed mainly of a very large number of corpora lutea atretica, of the type figured in Part II (Plate 7, fig. 2), but they present no other features of interest.

NVS 13 had eight regular cycles observed and at the end of the last of these the ovaries were retracted as in NVS 12. The next cycle had a duration of only 8 days, which was noticeably less than that previously found. The next four cycles were (11)–(9)–(10)–(10) days respectively in length, ¼ B tint irradiation being given, half in the first and half in the second of these cycles. After the end of the fourth of these cycles no further œstrous symptoms were observed for 43 days, the left ovary being removed 16 days before the copulation which ended this long " anœstrous " period. The subsequent œstrous history of the animals is summarised in the following cycle formula :—

(12)–(8)–(8)–13–(8)–(3)–(4)–(6)–7–5–5–8.

Autopsy was performed 3 days after the end of the last œstrous period. The difference in the history of these two animals after experimental examination is rather striking, but the definite, if irregular, œstrous history of NVS 13 makes it clear that the cessation of the cycle in NVS 12 was not an essential consequence of either the operative or irradiation treatment.

The ovaries of NVS 13 were composed of a large number of corpora lutea, probably atretica, and the old interfollicular and follicular tissue had become markedly luteal in nature.

VII.—*Discussion.*

The results described in the present paper confirm those previously arrived at. The small oocytes in primordial follicles degenerate and disappear completely within 24 hours of irradiation. Anovular follicles are formed from some of the smaller follicles by the degeneration of the oocytes within a day after irradiation of the pregnant or lactating adult, as was found previously in the non-parous adults (3).

In the adults described in this paper the formation of the tissues of the sterilized ovaries from the elements of the cortex, and not from a proliferation on the part of the germinal epithelium was clearly shown, and confirmed the previous results on adult animals (3). Further, the correlation existing between the luteal-like condition of this tissue and the stoppage of the œstrous cycle was observed in a number of cases (XVP and PC series). In one animal, XVP 7, persistent vaginal cornification was found again (see 3) to be associated with a highly vacuolated condition of this tissue.

It is remarkable that in no case was sterilization found to be complete under 40 days after irradiation when adult, although this was found to occur in a few cases among the animals irradiated at 3 weeks old (1). Altogether 108 animals irradiated when adult are described (see Part III of the present paper) of which 24 were killed at varying times less than 40 days after irradiation. All these were found to contain one or more follicles. Fifty-nine of the remaining 84 killed 40 days or more after irradiation were completely sterilized. Coupled with this is the fact that adult animals became pregnant up to a week after irradiation and even produced a litter, although on subsequent examination, more than 40 days after the dose of X-rays was administered, the ovaries were found to be completely sterilized.

These remarkable phenomena appear to admit of only one explanation. The lethal action of the X-rays on the ovarian oocytes appears to be inversely proportional to their size. This results, with the dose employed, in the complete destruction of all the small oocytes and of the majority of the large ones. Those large ones which do not degenerate immediately appear to remain normal. Some of them subsequently degenerate, as occurs frequently in the normal ovary, others mature and are ovulated. These appear to be capable of fertilization and normal development. The total number of such unaffected large oocytes appears, however, to be so small that they are soon all used up, either by degeneration or ovulation, and in most cases none remain by 40 days after irradiation.

It is therefore clear that no new oocytes are formed after irradiation, as was pointed out previously. This is more remarkable now that it is known that certain oocytes can live, mature and subsequently develop after fertilization in the X-rayed animal. It is, consequently, more difficult to suppose that irradiation has permanently divested the ovary of the power which it is said by some to possess, of forming new oocytes during adult life. On the contrary, these facts *afford strong evidence that no formation of new oocytes normally* takes place in the adult ovary.

The corpora lutea.—The material described shows clearly that the functional life of the corpora lutea of ovulation, gestation or lactation is not increased after irradiation, and that degenerative changes become apparent in them at the same age as in the normal animal. On the other hand, degeneration itself is much less rapid after irradiation, and the remnants of old corpora lutea persist for a much longer time in the ovary and become almost permanent constituents. Long and Evans (5) suggested that the presence of functional corpora lutea, especially of gestation and lactation, accelerated the degeneration of previously formed ones. The presence of maturing follicles may have a similar effect. The persistence of the corpora lutea in the sterilized ovaries is probably due to the lack of competition of growing follicles and functioning corpora lutea. *In any case these results show that the presence of œstrin in* the ovary is not the accelerating agent in the degeneration of the corpora lutea, although it may be responsible for initiating it.

VIII.—*Summary.*

1. The present paper deals with 64 animals irradiated during pregnancy or lactation. The results confirm those previously obtained on non-parous adults (3).

2. The absence of neo-formation of oocytes after X-ray sterilization and its significance is discussed.

3. The degenerative changes in the corpora lutea start at the same age in the sterile as in the normal ovary. They proceed more slowly, however, and the old corpora lutea become practically permanent components of the sterile ovary. *This is attributed to the absence of competition with maturing follicles* and new corpora lutea.

4. Two cases of compensatory hypertrophy of the sterilized ovary after unilateral ovariotomy are described.

5. Two cases of regenerated ovarian tissue sterilized by X-rays are described. Both resemble the tissues of the sterilized normal ovary.

6. Some miscellaneous experiments on the sterilized ovary are described.

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DESCRIPTION OF PLATE 23.

GUIDE LETTERS.—A.F., Anovular follicle. B.C., Blood clot. C.L. Corpus luteum. D.M., Degenerate multinucleate cell masses. I.F., Interfollicular tissue. D.O., Degenerating oocyte. T.A., Tunica albuginea.

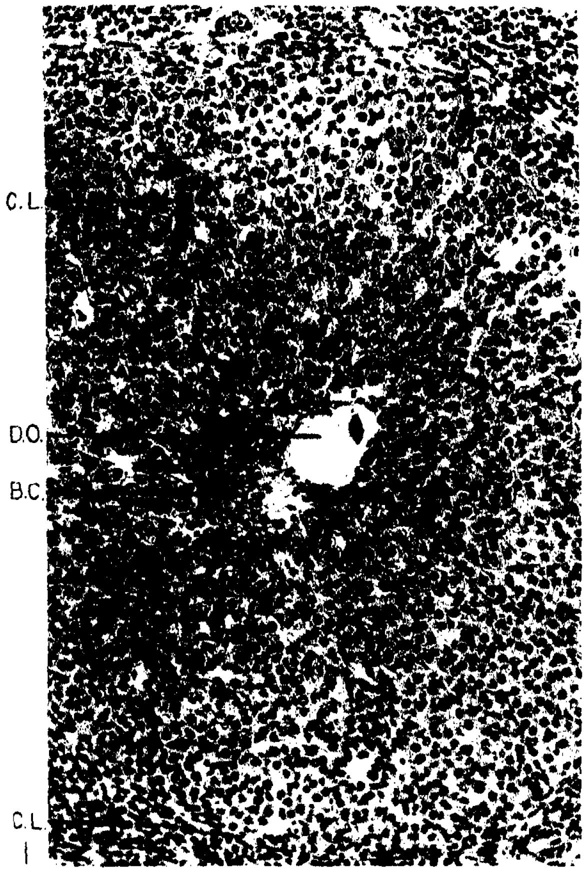
FIG. 1.—PC 26, showing corpus luteum atreticum with the central blood clot and an enclosed degenerating oocyte containing a crystalloidal body.

FIG. 2.—XVP 7, showing for comparison the vacuolated interfollicular tissue and part of a corpus luteum.

FIG. 3.—XVP 3, showing for comparison the luteal-like interfollicular tissue and part of a corpus luteum.

FIG. 4.—PC 14, showing anovular follicles in the periphery and masses of multi-nucleate cells formed as degenerate products of the luteal-like cells of the interfollicular tissue.

We are indebted to Mr. F. J. Pittock for the photomicrographs reproduced in these plates.



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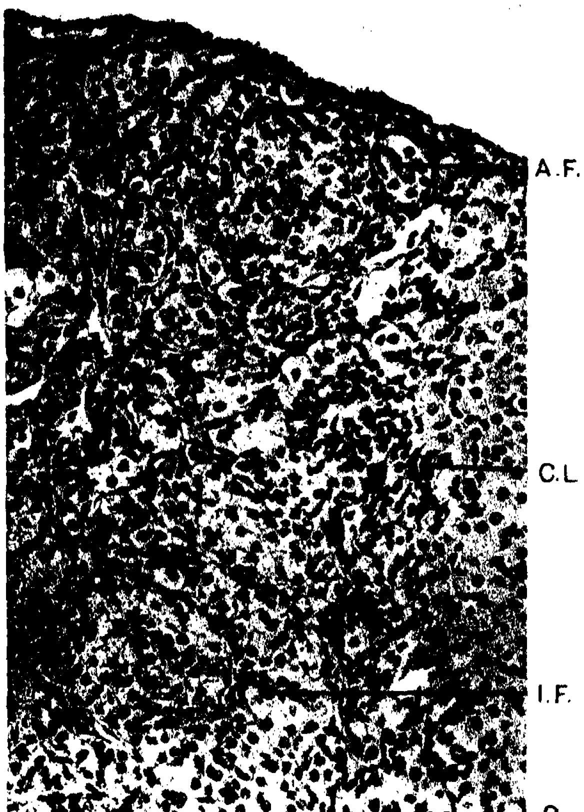

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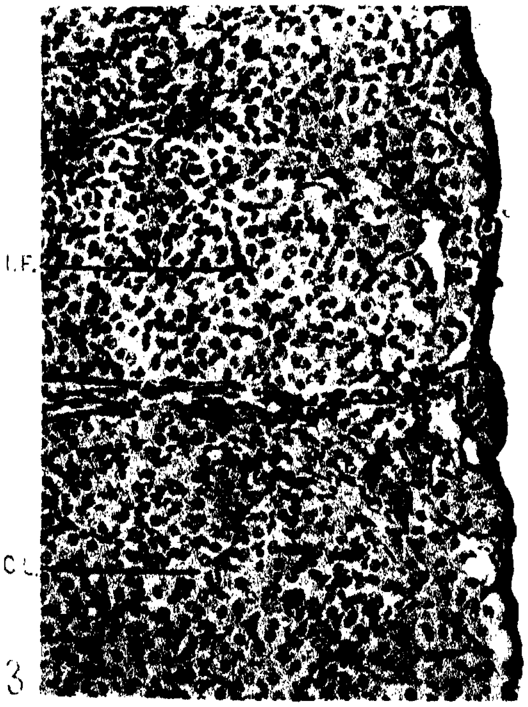

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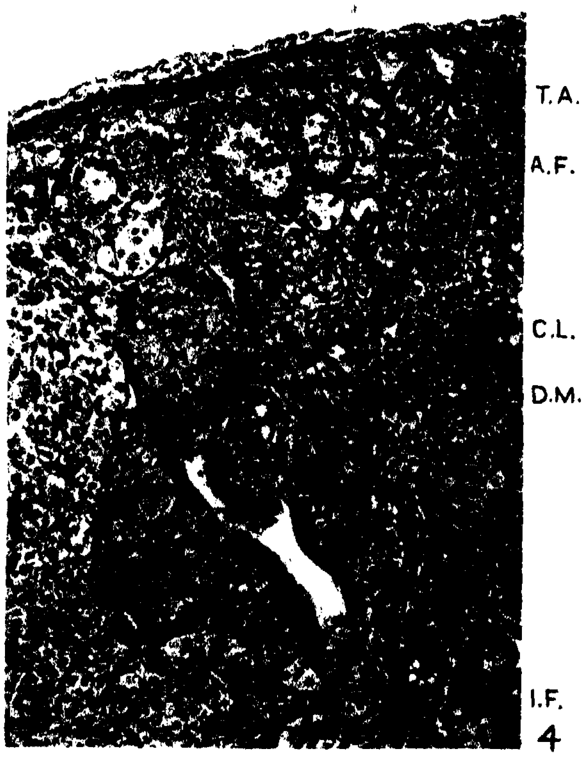

4

Studies in the Microchemistry of the Cell. I.—The Chromatin Content of Normal and Malignant Cells, as demonstrated by Feulgen's "Nuclealreaktion."

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(Communicated by J. A. Murray, M.D., F.R.S.—Received December 3, 1927.)

(From the Laboratories of the Imperial Cancer Research Fund.)

[Plates 24, 25.]

Since Feulgen introduced his "Nuclealreaktion" for the demonstration of nucleoproteids, several papers have been published by Continental workers recording the results of the application of this method to a wide range of animal cells. The general consensus of opinion is that the method provides a precise microchemical colour reaction for thymus-nucleic acid protein complexes. No other cell structures, or inclusions of any kind are stained, when the correct procedure is employed.

Chemical Basis of the "Nuclealreaktion."

The "Nuclealreaktion" is based upon Schiff's test for aldehydes (3). As a microchemical method it depends upon the presence of an aldehyde group among the products of a partial hydrolysis of thymus-nucleic acid, the nucleic acid of animal nuclei. When thymus-nucleic acid is subjected to a mild hydrolysis, and the resulting products are treated with fuchsin-sulphurous acid, there results therefore an intense purple coloration. This colour substance is insoluble in water, and only very slightly soluble in alcohol.

The partial hydrolysis of thymus-nucleic acid separates off the purine bases, adenine and guanine, leaving thymic acid, a more resistant and ill-defined substance. According to Feulgen, it is the thymic acid which contains the aldehyde group and gives the Schiff reaction with fuchsin-sulphurous acid. Where the term "chromatin" is used in this paper, it is to be understood, therefore, that it refers only to the chromatin of animal nuclei.

Technique of the Reaction.

In practice, with sections, the method is carried out in three stages. Slides of suitably fixed material are brought down to water. Then, firstly, the hydrolysis is effected in HCl at a temperature of 60° C. for about five minutes. Secondly, the slides are transferred to fuchsin-sulphurous acid. Thirdly, excess of the stain is removed by a thorough washing in water containing excess of SO_2.

Hydrolysis of the sections sets free the aldehyde group of the thymus-nucleic acid. This gives rise to the purple coloration when brought in contact with fuchsin-sulphurous acid. Washing in sulphurous acid removes all the stain, the SO_2 preventing the formation of the basic fuchsin from the fuchsin-sulphurous acid. When, therefore, the sections are brought to water, preparatory to mounting, all the fuchsin-sulphurous acid has been removed from the tissues, and what was previously chromatin is represented by a bright purple or violet coloration.

Preparation of the Reagents.

(*a*) *The Acid used for the Hydrolysis*—82·5 c.c. of HCl (S.G. 1·19) are dissolved in 1 litre of water.

(*b*) ***Fuchsin-Sulphurous Acid.***—200 c.c. of distilled water are brought to boiling. 1 gm. of basic fuchsin is then added and the water well stirred. After the fuchsin has dissolved the solution is allowed to cool to about 50° C., when it is filtered. Then 20 c.c. of N . HCl are added. When the liquid has further cooled to about 25° C., 1 gm. of anhydrous sodium bisulphite is dissolved in it. This results in the liberation of SO_2, and the fuchsin is slowly decolorised. It should be allowed to stand for 24 hours at least. As soon as a yellow colour is attained it is ready for use. It should be kept in a well-stoppered bottle in the dark.

(*c*) *The Water containing an Excess of* SO_2.—To 200 c.c. of distilled water is added 10 c.c. of a 10 per cent. solution of anhydrous sodium bisulphite, and then 10 c.c. of N . HCl. This water should only be used so long as it smells strongly of SO_2. It must be renewed frequently.

Carrying out of Reaction.

1. *Fixation.*—Alcohol, alcohol acetic, sublimate, or sublimate acetic can be used. Feulgen recommends sublimate acetic, consisting of a 6 per cent. solution of mercuric chloride, to every 100 c.c. of which 2 c.c. of glacial acetic acid are added. I find this fixative gives the best results for general cytological purposes. Small pieces of tissue are fixed for 24 hours, then washed in running water, brought up through the alcohols to xylol, and embedded in wax. My sections have then been cut at 5 μ. They are brought to water in readiness for the hydrolysis. It is advisable to leave sections for some hours,

or even overnight, in 90 per cent. alcohol before carrying out the hydrolysis, as this hardens the tissues and reduces to a minimum the distorting action of the warm acid.

2. *Hydrolysis and Colour Reaction.*—The acid used for the hydrolysis must be kept at 60° C.; the other reagents are used at room temperature.

Verne (8) suggests having the reagents contained in tubes arranged serially. I have adopted this method as being a very convenient one in practice. For the hydrolysis I have used a beaker partly filled with acid, and kept warm by a luminous Bunsen flame. It is essential to have a thermometer dipping into the acid in order to enable one to maintain the temperature practically constant. A slight fluctuation of a degree or so either way does not affect the result. The general arrangement of the vessels with their contained reagents, together with the length of time slides should be kept in each, is as follows :—

Slides brought down to water are transferred to tube 1, then to the beaker, and so on.

Tube 1 →	Beaker →	Tube 2 →	Tube 3 →	Tube 4 →	Tube 5 →	Tube 6 →	Tube 7 →	Tube 8
HCl	HCl at 60° C.	HCl	Distilled water	Fuchsin-sulphurous acid	Water containing free SO_2			Distilled water
1 min.	4–15 min., usually 6 mins.	Few seconds	Just rinse	Up to 1½ hrs.	Move slides about in each tube 1–2 min.			1–2 min.

I find that the length of time that the slides remain in fuchsin-sulphurous acid can be greatly curtailed. With most of the material with which I have worked a purple coloration of the chromatin is evident almost immediately, and is intense after a few minutes.

The contents of tubes 3 and 8 should be changed each time. Tubes 5, 6 and 7 should be refilled when they no longer smell strongly of SO_2.

Counterstaining.—Since only chromatin is stained by this technique, it is usually desirable to employ some counterstain. Wermel (9) has suggested the use of light green. It affords a useful contrast to the purple chromatin staining. I find it convenient to stain as follows: After the slides have been finally washed in distilled water (in tube 8), they are dipped for a few seconds in a watery solution of light green. Excess of the stain is washed off with distilled water, which is then blown off the slides. Absolute alcohol is then poured on the sections, and used for their differentiation. They are finally cleared in xylol and mounted in balsam. The appearance of various types of cells after this treatment is shown in figs. 1–9 (Plate 24).

Previous Work.

The method has been applied to a wide range of animal tissues. Berg (1) has worked with tissues of the salamander, rat, rabbit, dog and man, while Wermel (10) has found the reaction to apply to cells of *Hydra, Helix, Loligo, Periplaneta, Lymantria, Astacus, Rana, Bufo, Axolotl*, rat, cat, dog and man. Nothing but chromatin was stained. Wermel also found that in the nerve tissue of the dog and cat the Nissl granules were not coloured. According to this worker an improvement in the technique results from using, instead of fuchsin-sulphurous acid, monoaldehyde products of this reagent.

Berg (1) has made a special investigation into the action of the hydrolysis upon the tissues fixed in corrosive acetic. He found that a certain amount of distortion was produced by the action of the warm acid. Nuclei might become slightly swollen. Some chromatin particles might become lost, or might undergo swelling. The nucleolus could also become enlarged. Once the chromosomes were formed, however, they were not altered by the hydrolysis. The nuclei of some material, which after fixation in corrosive sublimate had remained for 12 years in 85 per cent. alcohol, were not altered structurally by the action of the hydrolysis. Similarly, Zenker-Formol fixed material was not injured by the warm acid, although the subsequent colour reaction was weaker.

In connection with this criticism of Berg's, I should point out that I have found little indication of the injurious results which he describes and figures. I have not worked with the same tissues as he has, but he figures marked alterations in the follicle cells of the rat ovary. In my sections of oöcytes of the mouse surrounded by follicle cells, the nuclei of the latter show no indication of the vacuolation and enlargement of the nucleolus which Berg figures. It seems probable, therefore, that Berg's sections had not been sufficiently hardened in alcohol before the hydrolysis was carried out.

Up to the present the " Nuclealreaktion " has only been applied in a few cases to the many special problems of cytology, for the solution of which a satisfactory microchemical test for chromatin is demanded. Koch (4) has used the method for the study of the oögenesis of Chilopods. He was able to demonstrate chromatin in the oögonia and in the follicle cells, but not in the growing oöcytes. It was concluded, therefore, that during the growth of the oöcyte the chromatin undergoes a profound chemical change.

Roskin and Grünbaum (7) sought to ascertain by the method whether blood platelets contained nucleoproteids. They carried out the test with the blood of mice suffering from a trypanosome infection. They found no chromatin in the blood platelets, although it was demonstrable in the nuclei of the trypanosomes and of the leucocytes. Roskin (6) has also applied the method for the study of the nuclei of a mouse carcinoma. He points out that the chromatin which is stained a violet colour tends to lie against the nuclear membrane. The prophase nucleus contains a spireme structure, which is apparently made up of a central core covered with chromatin. Two types of degenerate nuclei occur. In one type there is considerable accumulation in the nucleus (" hypernucleiner Kern "); in the other type there is very little chromatin (" hyponucleiner Kern "). The diminution in chromatin content is the result of its having been extruded into the cytoplasm. Roskin adds that " besides these structures (extruded chromatin) in the plasma still other little rod-like inclusions were found, which also give the reaction for nucleic acid. I have not succeeded in determining the nature and origin of these structures."

Personal Observations.

I have carried out the " nucleal " reaction with various tissues of the rat and mouse, and also with the ovotestis and hepatopancreas of the mollusc *Limnæa stagnalis*. Material fixed in alcohol, corrosive acetic, and alcohol acetic has been used. The corrosive acetic fixation recommended by Feulgen gave the brightest staining. All sections were counterstained with light green.

Gametogenesis.—It was possible to detect chromatin by the reaction in the oöcytes of the mouse. A young oöcyte is represented in Plate 24, fig. 1. Its nucleus contains, in addition to the small granules of chromatin, a considerable amount of material which stains with the light green. The nucleolus contains no chromatin, although small granules frequently lie in contact with it. During the growth of the oöcytes the chromatin does not appreciably increase in amount. Fig. 2 shows an older oöcyte. Although the non-chromatinic material has increased considerably, the chromatin is represented by a few granules, three of them being closely associated with the nucleolus. When the nucleus has broken down and the chromosomes are formed for the first reduction division, no chromatin can be distinguished in the cytoplasm; but the chromosomes themselves stain intensely.

During oögenesis of *Limnæa stagnalis* scarcely any chromatin is distinguishable in the nucleus by Feulgen's method. A faint purplish coloration is to be seen in some of the nuclei, so that I am inclined to believe that the chromatin is so finely dispersed in the nucleus as to render its demonstration impossible by this method. It may be, of course, as Koch has suggested, that during oögenesis the chromatin undergoes profound chemical changes.

During growth of the oöcyte of *Limnæa* the nucleolus becomes enlarged. With the ordinary stains it is first oxyphil, then, later, partly oxyphil and partly basophil (5). Thus with methyl-blue-eosin the nucleolus at first stains red, then partly red and partly blue. Portions of the red-staining nucleolus are extruded into the cytoplasm. By Feulgen reaction no chromatin can be detected in either the oxyphil or basophil staining nucleoli. It might be pointed out here that sections stained with methyl-blue-eosin, and others subjected to the " nucleal " reaction, are remarkably alike as far as general histological features are concerned, so that the objection that the hydrolysis induces artefacts is, with this material, not tenable.

Spermatogenesis.—In both the Mollusc material and in the rat and mouse testis, the sperm heads stain intensely with the " nucleal " reaction. At those stages of spermatogenesis when the chromosomes are stretched, the reaction is only faint. It becomes progressively brighter with condensation to form the chromosomes. From the staining reaction it appears that the chromosomes are composed of at least two substances, one of which gives the Feulgen reaction and another substance, or more probably substances, which stain with light green. Fig. 11 (Plate 25) is a photomicrograph of a portion of the rat testis, showing the intense staining of the sperm heads and the lighter staining of the chromosomes at the prophase of meiosis.

Epithelium of Epididymis of Rat and Mouse.

These cells were examined especially because in them various types of nuclear extrusions into the cytoplasm have been described. A binucleate cell is shown at fig. 3, and a single nucleus from an adjoining cell at fig. 4. In fig. 3 a sperm head is included at the top of the cell for comparison. I find no indication of chromatin being extruded into the cytoplasm in the material which I have examined. It will be noticed in the figures that although the nucleolus is often surrounded by chromatin, none is actually contained within it.

Gland Cells.

The method has been found to give good results with the pancreas, thyroid and adrenal of the rat, and with the hepatopancreas of *Limnæa*. The large multilobed nuclei of the Mollusc contain a great deal of chromatin, as do also the nurse cells of the oöcytes. The gland cells of the rat and mouse, however, only contain the average amount.

Many writers have described extrusions from the nucleus, and loss of chromatin during secretory activity. Cramer (2) has shown that the cells of the medulla of the adrenal are stimulated to secretory activity by exposure to cold, and after functional activity the nuclei are considerably altered. Fig. 10 (Plate 25) shows a portion of the adrenal medulla of one of Dr. Cramer's experimental animals. The cells in the centre of the photograph are normal. They have scattered granules of chromatin. Immediately below these nuclei are four nuclei in the exhausted state. They are smaller and irregular in outline. They have lost a part of their contents, but not chromatin, since they give a brilliant coloration after Feulgen's reaction.

In the other gland cells which I have studied I find alteration in the size of the nucleus and in the amount of nucleolar material, but the amount of chromatin does not seem to alter to any appreciable extent.

Tumour Cells.

I have been unable to correlate any relationship between the amount of chromatin of a tumour-cell nucleus, and the rate of growth of the tumour. In sections through a tar tumour and the neighbouring skin no difference is apparent in the amount of chromatin in the nucleus of the normal and of the malignant cells. When the nuclei are larger, the chromatin is more scattered.

In most of the tumour cells examined the chromatin occurs as scattered granules, as shown in the case of the spindle-celled sarcoma (37 S) in fig. 5. The large nucleoli which are characteristic of many tumours contain no chromatin except as granules around their periphery. The degenerate nuclei which I have examined are much shrunken up, as shown in fig. 6. The chromatin particles run together, and the whole nucleus stains intensely. Scattered granules of chromatin as well as fragments of the nucleus occur in the cytoplasm.

The "Bonne" tumour which was examined shows great variation in the size of the cell nuclei. Three cells are shown in figs. 7, 8 and 9. Fig. 7 shows a prophase nucleus. The chromosomes are formed and are stained intensely. It will be observed that the nucleolus remains quite distinct from them. Fig. 9 is one of the smaller cells and fig. 8 one of the giant cells which are formed either by fusion of smaller nuclei, or as the result of irregular cell division. Such giant cells contain relatively large particles of chromatin.

Nuclear extrusion is a marked feature of some tumours. In an adeno-carcinoma (27 of this laboratory), where the process is to be seen, it was found that it was not chromatin but nucleolar material which was extruded. In the same way in tar tumours, where keratinisation occurs, it is principally nucleolar material which is extruded and forms keratohyalin.

Summary.

1. According to Feulgen, when sections of suitably fixed material are submitted to a mild hydrolysis, treated with fuchsin-sulphurous acid, and then thoroughly washed in sulphurous acid, the nucleoproteids of animal cells are stained an intense purple or violet colour. On the basis of this conception the following conclusions are drawn regarding the thymus-nucleic acid protein complexes (chromatin) of animal nuclei.

2. During oögenesis in the rat and mouse there is no increase in the chromatin content of the nucleus (figs. 1 and 2). When the germinal vesicle breaks down to form the chromosomes, no chromatin is extruded into the cytoplasm.

3. Neither the oxyphil- or the basophil-staining nucleoli of the oöcyte of the Mollusc, *Limnæa stagnalis*, contain chromatin.

4. The sperm heads of both *Limnæa* and of the mouse and rat stain intensely (fig. 11). At those stages of spermatogenesis when the chromosomes are stretched, the reaction is only faint, but becomes progressively brighter with condensation. There are indications that the chromosomes contain other substances in addition to chromatin.

5. No chromatin extrusion was observed in epithelial cells of the epididymis (figs. 3 and 4).

6. After secretory activity the nuclei of gland cells often become shrunken and irregularly lobed. This is well seen in the medulla of the adrenal, following exposure to cold. Such exhausted nuclei still stain brightly, so that it appears that it is some substance other than chromatin which is lost during functional activity (fig. 10).

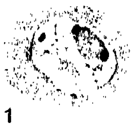
7. It was not found possible to demonstrate any relationship between the amount of chromatin in a tumour-cell nucleus and the rate of growth of the tumour.

8. In sections through a tar tumour and the surrounding skin no difference was apparent in the amount of chromatin of the normal and of the malignant cells.

9. During cellular degeneration in the tumours examined the nuclei become shrunken. The chromatin runs together and the whole nucleus stains intensely (figs. 5 and 6).

10. The chromosomes of all the tumours examined stain intensely. During the prophase of mitosis it can be seen that the chromosomes are separate from the nucleolus (fig. 7).

11. The nuclear extrusions, which are well marked in some tumours, consist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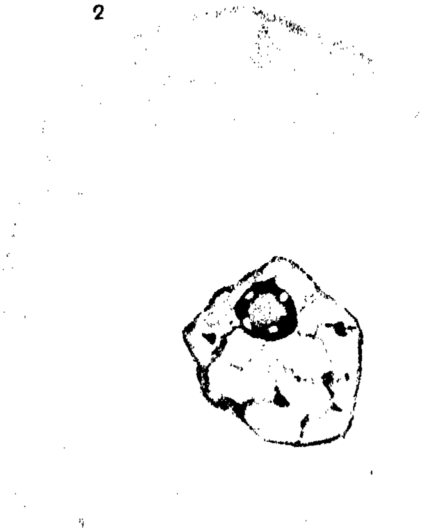
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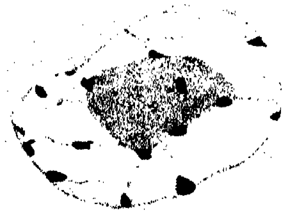
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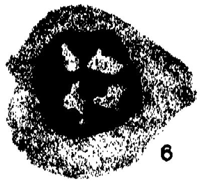
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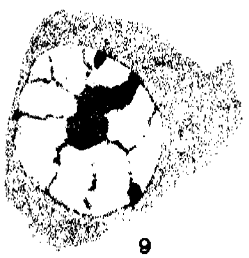
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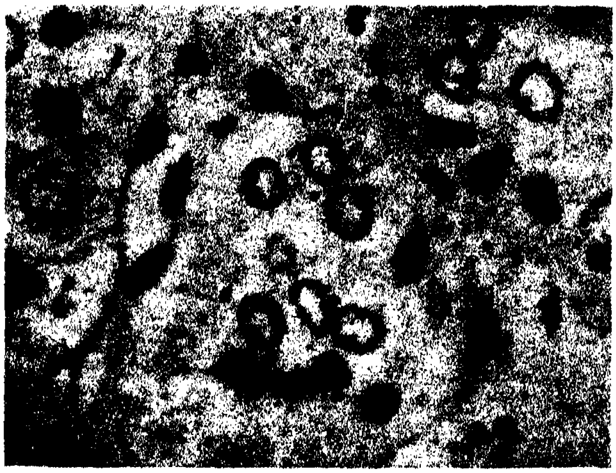

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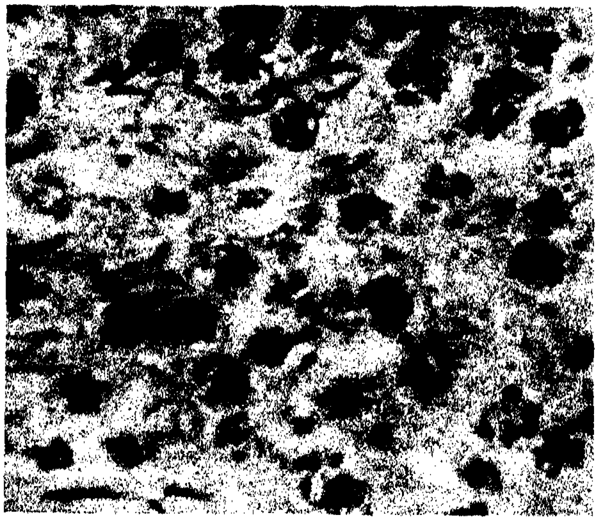

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of nucleolar material and not chromatin. It is principally nucleolar extrusions which give rise to keratohyalin during cornification.

12. Some large nuclei contain apparently the same amount of chromatin as smaller ones, but the giant nuclei which occur in some tumours and are formed either by irregularities of cell division or by the fusion of smaller nuclei contain large masses of chromatin (figs. 8 and 9).

DESCRIPTION OF PLATES 24–25.

PLATE 24.

Appearance of various types of cells after Feulgen's "Nucleal" reaction, and counterstaining with light green. Figs. 1 and 2 are drawn to a lower scale than the others. The actual magnification is indicated by the two scales.

In all the figures, which are reproduced from coloured drawings, the chromatin —purple in the preparations—is shown black. The green counterstain is here represented grey.

Figs. 1 and 2, oöcytes of the mouse; fig. 3, binucleate cell of epithelium of epididymis; fig. 4, single nucleus from a neighbouring cell; fig. 5, normal cell of the spindle-cell sarcoma (37 S); fig. 6, degenerate cell from the same tumour; fig. 7, prophase of mitosis in the Bonne tar sarcoma; fig. 8, giant cell, and fig. 9, normal size cell, both from the same tumour.

PLATE 25.

Photomicrographs of preparations stained as in Plate 24. The photographs were taken with the Leitz "Mikam" camera through a green filter, so as practically to block out the green counterstain, and at the same time to accentuate the purple coloration of the chromatin.

Fig. 10, cells of the medulla of the adrenal of the rat after exposure to the cold; fig. 11, testis of the rat.

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Cytological Studies on the Viruses of Fowl-Pox and Vaccinia.

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(Communicated by J. A. Murray, F.R.S.—Received December 3, 1927.)

(From the Laboratories of the Imperial Cancer Research Fund.)

[Plates 26–30.]

CONTENTS.

	Page.
1. Introduction	406
2. Virus Bodies in Fowl-pox (epithelioma contagiosum)	407
3. Virus Bodies produced in the skin of the Chick by the Vaccinia Virus	408
4. Vaccinia Virus Bodies in Epidermal Cells of the Cornea of the Chick	410
5. Vaccinia Virus Inclusions in the Epidermal Cells of the Cornea of the Rat	411
6. Vaccinia Virus in Tumours and in the Testis	414
7. General Conclusions	415
8. Summary	415
9. Bibliography	417

1. *Introduction.*

Although, as is well known, the ultra-microscopic viruses are invisible in histological preparations, yet characteristic bodies occur within certain of the cells of animals infected with such organisms. The origin and nature of these so-called "virus bodies" has been the subject of much controversy. By some they have been regarded as the actual parasite, or at least as phases in its life cycle. To von Prowazek they were dual in character consisting of micro-organisms embedded in material produced by the reaction of the cytoplasm of the infected cell. Still other observers regarded such bodies as products of cellular disintegration. In a former paper (Findlay and Ludford (1926)) we have referred to the various views held by writers in this field and have made a survey of the literature of the subject in the form of a pictographic review. We shall, therefore, only mention previous work in this field, in so far as it directly concerns our personal observations.

A conspicuous fault of much of the earlier work has been the unsatisfactory histological technique employed, especially the nature of the fixative. The earlier cytological work was carried out with fixatives, which although satisfactory for the subsequent demonstration of nuclear structures were very destructive to the cytoplasm. This is particularly unfortunate since most of the virus bodies occur in the ground cytoplasm of cells. We have endeavoured to rectify this source of error in our work by employing fixatives, which have been proved to fix the cells in such a manner as to give an appearance as nearly as possible identical with their structure, as seen in the living cells.

The present communication is devoted to an account of the virus bodies produced in epidermal cells of the rat and fowl by the vaccinia virus, and the relationship of these to the inclusions produced in the fowl and pigeon by the virus of fowl-pox, or epithelioma contagiosum, which were described in a previous communication (Ludford and Findlay (1926)).

I am indebted to Dr. G. M. Findlay for the material on which these observations have been carried out.

2. *Virus Bodies in Fowl-pox* (*Epithelioma Contagiosum*).

Within epidermal cells of the skin lesions of fowls infected with the virus of fowl-pox there occur conspicuous virus bodies. The first definite indication of infection of an epidermal cell with this virus is the appearance of one or more minute vesicles. With progress of the infection these vesicles increase in number and become enlarged. At an early stage they become surrounded by a lipoidal cover. Within the vesicle itself granules make their appearance commencing at the immediate periphery. The various vesicles tend to coalesce so as to produce, ultimately, a single large virus body composed of either minute granules or vesicles.

One strain (C) of the fowl-pox virus which was investigated was found to produce in the skin of the pigeon exactly the same type of virus body. The histological picture of the lesion was identical with that in the skin of the chick. The virus bodies originated in the same way, and underwent the same development. Scarification of the cornea of the chick and inoculation with fowl-pox virus results in exactly similar changes to those occurring in the skin, but in epidermal cells of the rabbit, and in corneal cells of the rabbit and rat, none of the three strains of virus examined gave rise to any form of cell inclusion.

3. *Virus Bodies produced in the Skin of the Chick by the Vaccinia Virus.*

Our study of the fowl-pox virus bodies led us to investigate the effect of the vaccinia virus on the skin of the chick. In these experiments, small chicks were inoculated on the side of the head, the feathers being pulled out. The lesions which made their appearance were extirpated and fixed according to the Schridde and modified Kopsch methods previously described (Ludford (1924^2, 1925)).

Sections of the lesions appeared identical with those caused by the fowl-pox virus. There was hypertrophy of the epidermis and the usual well marked reticulo-endothelial dermal reaction. Further, virus bodies exactly similar to those of fowl-pox developed in the epidermal cells. Figs. 1 to 5 (Plate 26) show the appearances presented by these virus bodies in unbleached Schridde preparations, which had been stained with iron-alum-hæmatoxylin. Fig. 1 is a cell from the lowest layer of the epidermis. Amongst the mitochondria (*m*) are seen vacuoles (*vv*), which are the first clear indication that a cell has become infected. Fig. 2 represents a later stage in the development of the virus inclusions. Two of the vacuoles (*vb*) have become covered with a lipoidal coat. Two smaller ones are still colourless. Most of the mitochondria (*m*) of this cell retain their filamentous form, although there is indication of fragmentation.

The virus bodies (*vb*) of fig. 3 are larger, while the mitochondria are fragmented, and are beginning to disappear. At the stage of infection, shown in fig. 4, the larger virus bodies are coalescing, but numerous smaller ones are in process of formation. In the cytoplasm can be discerned a number of granules about the size of mitochondria. These vary in size from a minute dot, just on the borderline of visibility, to granules about the size of an enlarged mitochondrium, the latter being also the size of the smallest vacuole. The study of numerous cells exhibiting these appearances leads one to conclude that the minute granules only just discernible are the very first visible phase in the development of the virus structures. The successive stages in the development of the virus bodies according to this interpretation are indicated in fig. 4 as v_1 to v_7.

As with the fowl-pox so with the vaccinia virus the virus bodies after becoming enlarged and coated with lipoid coalesce to form the single large inclusion body; such bodies lie within cells of the most superficial layers of the epidermis of the skin lesions. Four cells in which the virus bodies are almost completely formed are shown in fig. 5. In each case these bodies (*vb*) are somewhat irregularly stained, so that darker and lighter areas are easily apparent. Three of the cells in this figure have in addition to large virus bodies, smaller ones in process of formation. The latter would, no doubt, ultimately become incorporated within the large body. This is probably what has happened in the fourth cell of this figure which has only one large virus body.

Since during the course of their development, the virus bodies become coated with a lipoidal substance, which is blackened in Schridde preparations, it is impossible to determine the nature of their interior in unbleached sections. Hydrogen peroxide was therefore employed to bleach sections before staining. Such bleached sections were stained by the iron-hæmatoxylin method, in the usual manner. Some were mounted after the usual differentiation, others were further decolorised until the stain had been removed from all parts of the cell with the exception of the virus bodies, this having been found to be possible in our former work on the fowl-pox virus bodies. After decolorisation the sections were stained with aniline acid fuchsin (Altmann's stain) by heating the stain on the slides until steaming occurred, then allowing to stand for 15 minutes. Differentiation was then carried out either in alcohol, or in an alcoholic solution of aurantia. In sections suitably stained by this method, the virus bodies were black to purple in colour. Nuclear structures and mitochondria were coloured red.

Three stages in the development of virus bodies, as seen in bleached and stained sections are represented in figs. 6 to 8. The cell of fig. 6 shows the earliest stage of infection. The mitochondria (*m*) are already fragmented. Towards the lower pole of the nucleus are a number of small virus vacuoles, amongst which are very minute granules. What are regarded as the sequence of stages in the formation of the virus bodies are indicated by the lettering v_1 to v_5, as in fig. 4, to which reference has previously been made. After the virus vacuoles have become enlarged, granules or vesicles make their appearance starting at the periphery, and ultimately coming to fill the whole vacuole. Figs. 7 and 8 show the contents of the virus bodies. Owing to their minute size it is impossible to determine with certainty whether the virus bodies are composed of granules or vesicles. In examining sections with an oil-immersion lens the slightest movement of the fine adjustment gives a vesicular appearance to what has previously looked granular, without altering perceptibly the optical resolution of the other larger cell structures.

In our former paper on the cytology of fowl-pox, we pointed out that a close relationship existed between the Golgi apparatus and the formation of the virus bodies. Exactly the same relationship exists with these virus bodies. The formation of virus bodies in cells in which the Golgi apparatus is impregnated by osmic acid is shown in figs. 9 and 13 to 16 (Plate 27). Fig. 9 shows a normal cell from the lowest layer of the epidermis of the chick's skin. The Golgi apparatus (GA) assumes the typical form of a closed network. Fig. 13 represents a cell from the infected area of the skin. Its Golgi apparatus (GA) is hypertrophied although as yet no virus bodies are visible. In the cell of fig. 14 are two virus vacuoles (*vv*), and one which has been coated with lipoid (*vb*). All three are closely associated with strands of the apparatus (GA). The next cell, fig. 15, has a single large virus body with strands of the Golgi apparatus on either side of it, while in the cell of fig. 16, the virus bodies are apparently surrounded by portions of the fragmented apparatus. This intimate relationship between the Golgi apparatus and the formation of the covering layer of the virus bodies rather suggests that this substance is formed by the cells as a secretion, comparable to the origin of secretion granules in contact with the apparatus in gland cells.

This study of the vaccinia virus inclusions in cells of the epidermis of the chick has shown, therefore, that both as regards their structure and their mode of origin, they are identical with the virus bodies of fowl-pox or epithelioma contagiosum.

4. *Vaccinia Virus Bodies in Epidermal Cells of the Cornea of the Chick.*

Scarification of the cornea of the chick with vaccinia virus results in a reaction similar to that in the skin. There is hypertrophy of the epidermal cells, and the same reticulo-endothelial reaction. Virus bodies are formed similar to those in the epidermal cells of the skin. Since infection of the cells is followed by fragmentation of the mitochondria, it is impossible to distinguish with certainty between granular mitochondria and the initial stages in the formation of the virus bodies. Fig. 17 shows an early stage of infection of a cell. Some of the mitochondria (*m*) are broken up, others remain filamentous. Three virus vacuoles (*vv*) are clearly distinguishable. Subsequently, the mitochondria become completely broken up, and their number begins to diminish. At the side of the nucleus there occur clusters of granules (figs. 18 and 19) amongst which it is impossible to distinguish mitochondria from the initial stages of the virus bodies. With progress of the infection, however, the granules enlarge to form vesicles. The latter become more numerous, increase in size and stain progressively more deeply with hæmatoxylin.

There is the same tendency towards coalescence, resulting in a single large virus body (fig. 21). In fig. 19 the various stages in the development of the virus bodies are indicated v_1 to v_5.

The same relationship exists here, also, between the formation of the virus bodies and the Golgi apparatus. The early stages of the process are shown in figs. 10 to 12 which are drawn at a higher magnification than the other cells. In fig. 10 the initial hypertrophy of the apparatus (GA) is to be seen. Fig. 11 shows virus vacuoles surrounded by the enlarged apparatus, while the cell of fig. 12 is filled with virus vacuoles, only fragments of the apparatus remaining.

It is important to emphasise at this point the essential similarity of the virus bodies produced by vaccinia in the epidermal cells of the skin and the cornea of the chick, because when we come to the study of the action of the same virus on the corneal cells of the rat we see that the inclusions are different, although, as we shall show later, it is possible to correlate certain features in their mode of origin.

5. *Vaccinia Virus Inclusions in the Epidermal Cells of the Cornea of the Rat.*

Scarification of the cornea of the rat with vaccinia virus is followed by well marked lesions in the epidermal layer. At the centre of the lesion the cells are killed, and this area of necrotic material is surrounded by a zone of hypertrophied epidermis the superficial layers of which present a cyst-like appearance. The cyst-like cavities are in most cases filled with a very finely granular material inside which polymorphs are often present. At other times the granular material is replaced by bacteria. Both in its mode of origin in the cells, and its accumulation in masses at the surface of the epidermis, this granular substance bears a striking similarity to the virus bodies of the chick skin. We shall describe the mode of formation of this material later.

Nucleolar extrusion is a common feature of the epidermal cells of the infected area. Apparently the nucleolus, or portions of it, become applied to the surface of the nuclear membrane and then pass out through it into the cytoplasm, see figs. 22 and 23 (Plate 28). Such extruded nucleolar material (*nx*) has been repeatedly confused with virus bodies. The so-called Guarnieri bodies are no doubt nucleolar extrusions, but they are in no wise specific reactions to infection with the vaccinia virus as they occur in many different kinds of cells under varying physiological and pathological conditions (Ludford (1924)), *e.g.*, they can be seen in the area of keratinization that is well marked in the regenerated epidermis subsequent to mechanical injury to the skin of mice. In cells which show this nucleolar extrusion, accumulations commonly occur of the finely granular substance, to which reference has already been made. This substance is marked (*g*) in figs. 22 to 24. It is shown perhaps most clearly in fig. 24, where it is scattered throughout a vacuole-like space in the cytoplasm.

The same process of nucleolar extrusion is to be seen in the cornea of the sheep scarified with sheep-pox. Figs. 25 to 27 show cells drawn from a preparation of the infected sheep's cornea, which was given to us by Prof. McIntosh. The nucleolar extrusions often come to lie within concavities in the surface of the nuclear membrane. When the nucleus is cut tangentially they then appear as is shown in fig. 25, marked GT. In sections at right angles to this plane they appear as shown marked GS. (Figs. 26 and 27.)

In epidermal cells immediately surrounding the centre of the lesion there occur peculiar accumulations of granules which Schütz (1925) has regarded as the specific virus bodies of vaccinia. Of these granules, he says, that they do not originate from extruded nucleoli or degenerate cell organs, but that they are true cytoplasmic structures arising as the result of infection of the cell by the vaccinia virus. The occurrence of such bodies on the margin of the injured area seems to suggest that they are restricted to the region of regeneration. These bodies are shown in figs. 28 to 31. They are first distinguishable as a small group of granules (G) at the side of the nucleus (fig. 9). They increase in number and spread throughout the cell, as is shown in figs. 29 to 31. No lipoidal sheath is formed around them and the individual granules fail to become vesicular.

It was at first deemed possible that these granules represented mitochondria. This view is untenable, however, for the following reasons :—

(*a*) During cellular degeneration (autolysis) the mitochondrial content of a cell becomes progressively reduced, until mitochondria can no longer be distinguished. Sometimes they become converted into fat droplets. These granules in contrast to the mitochondria increase in numbers with the progress of the infection. Corneal cells immediately surrounding the lesion contain numerous granules as shown in fig. 31, those further away contain few granules as in figs. 28 and 29.

(*b*) It frequently happens in resting cells, and also in cells beginning to degenerate, that the mitochondria fragment and become collected together around the sphere. In sections cut tangentially to the surface of the sphere the appearance is that of a cluster of granules. If, however, the plane of section goes diametrically across the sphere, then examination of the section shows the granular mitochondria grouped around a central clear area—the idiosomic, or sphere substance. During autolysis the sphere frequently enlarges so that this appearance is quite a striking feature of sections of degenerating tissues. The groups of Schütz's granules also differ from mitochondria in that they are fairly uniformly distributed throughout a portion of the cytoplasm which is distinguishable from the ground cytoplasm.

Although these granules seem definitely to be the result of infection of the cell they are unlikely to be the virus for they seem too large, and, in addition, they tend to become polymorphic, and to disintegrate. It might here be pointed out that vaccinia heated to 56° C. for 30 minutes did not produce any Schütz bodies, so they cannot be due to any foreign protein. Furthermore, they were not formed in autolysed epidermal cells of the cornea. We seem forced, therefore, to the conclusion that they represent the result of infection of the regenerating cells of the lesion.

The evidence that the finely granular substance which forms definite masses in the virus is very much stronger. Firstly, it occurs in masses in cyst-like cavities widely distributed throughout the lesion. The size of the particles is extremely minute, and they cannot be stained at all satisfactorily. Then again this substance seems to be formed in vacuoles, which arise in intimate relationship with the Golgi apparatus.

We have grouped together in figs. 32 to 41 (Plate 29) what are regarded as the various stages in the formation of the masses of this substance, such as occur in the superficial layers of the cells of the cornea. Fig. 32 shows a normal cell of this layer. Its Golgi apparatus (GA) is in the form of a closed network at the upper pole of the nucleus. In the cell of fig. 33 the apparatus is more spread out, and is beginning to break up. Such fragmentation as this occurs normally in all epidermal cells in the course of cornification. The process has been described previously in normal and pathological growth (Ludford (1924), and Ludford and Findlay (1926)).

The earliest pathological change is the appearance of vacuoles in the cytoplasm in contact with the Golgi apparatus, the polarity of which becomes reversed (*cf.* figs. 34 and 35). A similar reversal of polarity also occurs in epidermal cells of the skin of the chick subsequent to infection with the same virus. Attention is directed here to the essential similarity in the relationship of the virus bodies of the chick, and the vacuoles of the rat, to the Golgi apparatus (*cf.* in this connection figs. 32, 33, 34 and 35 (rat cells) with figs. 9, 13, 11 and 14 (chick cells)).

Returning now to the further changes in the vacuoles of the cells of the rat cornea, in fig. 35 it will be noticed that the vacuoles are more numerous than in fig. 34. At the same time the apparatus (GA) has become broken up into rodlets and granules. Subsequently the vacuoles seem to run together as do the virus vacuoles in the skin of the chick. Within the large vacuoles formed by this coalescence appears the finely granular substance (*g*), to which reference has already been made. As the cells pass from the lowest layers of the epidermis of the cornea toward its surface, the vacuoles, such as that shown in fig. 36, appear to increase in size. The Golgi granules eventually disappear, and the cell is reduced to the form of a large vacuole (*v*) containing the finely granular substance (*g*), surrounded by a covering layer of cytoplasm (figs. 39 and 40). Ultimately at the surface of the cornea we get the large cyst-like cavity filled with granular substance. The masses of this substance often present a finely vesicular appearance as is indicated in fig. 41.

It is seen, therefore, that both with regard to origin, and also mode of development, the vacuoles of the rat cornea and of the chick skin are essentially similar. The fully formed structures in the two animals differ, firstly, in the presence of the intensely osmophil covering substance in the chick and its absence in the rat; and, secondly, in the more minute size of the particles in the case of the rat. The latter difference may simply be due to the fact that we are observing clusters of the finer granules in the case of the chick virus bodies.

6. *Vaccinia Virus in Tumours and in the Testis.*

It will have been noticed in the previous description of the vaccinia virus bodies that they have all occurred in epidermal cells. We have observed none in any other type of cell, although the reticulo-endothelial reaction of the sub-epidermal tissues which Ledingham (1924) has described is a conspicuous feature of our preparations. To find whether virus bodies were formed in all types of cell we injected the vaccinia lymph into several transplantable tumours, the prototypes of which were epidermal cells. In no case were virus bodies seen in the tumour cells. The centre of the inoculated area became necrotic after a few days. This area was surrounded by tumour cells showing all stages of autolysis and necrosis. This zone of cellular degeneration was characterised by cells with considerable vacuolation of their cytoplasm. In Schridde preparations bleached with hydrogen peroxide, and then stained with iron-alum-hæmatoxylin, the cells appeared as in figs. 42 to 45 (Plate 30). (Mouse 2146, I.C.R.F.)

Fig. 42 shows two cells in which the formation of vacuoles (*d*) has just begun. The cells of figs. 43 to 45 contain more and larger vacuoles. In some cells as fig. 45 they are surrounded by densely stained cytoplasm. There seems little doubt that such appearances as these are simply due to degenerative changes in the cytoplasm. Portions of the same tumours were allowed to undergo autolysis in normal saline and then examined. A similar vacuolation was observable in these controls. We can conclude therefore that, in the case of the tumours we worked with, no virus bodies were formed in the cells although the toxin of the virus exercised a destructive influence on the cells.

The virus was also injected into the testes of rats. Here again there was a general pathological action upon the cells, but no virus bodies were seen. Figs. 46 to 51 show characteristic pathological changes in the spermatocytes. Although mitochondria disappear from the cells, and the nucleus undergoes chromolysis (figs. 49 and 50) and becomes pycnotic (fig. 47), the Golgi apparatus (GA) frequently persists to a late stage of degeneration (figs. 47, 48 and 51). Vacuolation of the cytoplasm is a common feature of both Schridde and Kopsch preparations. Fusion of degenerating cells to form a single large multinucleate mass (plasmogamy) is of common occurrence (fig. 51).

7. *General Conclusions.*

Cytological study of the vaccinia virus has shown :—

(1) That the virus bodies produced by vaccinia in epidermal cells of the skin and cornea of the chick are exactly the same as those of fowl-pox. Their structure is the same, they originate in the same manner and undergo the same development.

(2) That the vaccinia virus produces in epidermal cells of the cornea of the rat inclusions closely resembling those it forms in the cornea of the chick, but differing fundamentally in the absence of the covering osmophil substance, which is so characteristic of the virus bodies of the chick. There are also formed in cells immediately surrounding the centre of the vaccinia lesion, clusters of granules (Schütz's granules) which are the result of the infection of cells of the regenerating epidermis.

(3) The vaccinia virus can be transmitted from the rat cornea to the skin of the chick where it then results in the formation of the characteristic virus bodies. Vaccinia virus from the chick brings about the same result in epidermal cells of the rat cornea.

(4) One strain of fowl-pox (C) was found to produce in epidermal cells of the pigeon the same type of virus bodies as in the chick but was without action on epidermal cells of the cornea of the rat. Two strains of fowl-pox (A and B) produced virus bodies in the chick, but not in the pigeon. Vaccinia virus did not form any virus bodies in the skin of the pigeon.

8. *Summary.*

The Virus Bodies produced in the Skin of the Chick by the Vaccinia Virus (figs. 1 to 21).—Virus bodies are believed to arise in the following manner (see especially figs. 4 and 6). A minute granule (v_1) becomes progressively larger (v_2, v_3), and assumes a vesicular form (v_4). Many such vesicles arise in the cells. They increase in size (v_5, v_6, v_7), become covered with a strongly osmophil substance, and fuse to form one or more large bodies, inside which appear numerous granules or minute vesicles.

During the course of their early development such virus bodies are intimately associated with the Golgi apparatus of the cell (figs. 9 to 16). Exactly the same kind of virus bodies are formed in the epidermal cells of the cornea of the chick as in the epidermal cells of its skin (figs. 17 to 21). Both as regards their structure and origin and development these virus bodies are identical with the virus bodies of fowl-pox or epithelioma contagiosum.

One strain of fowl-pox (C) which was studied was found to produce exactly the same kind of bodies in the skin of the pigeon. Two other strains (A and B) produced virus bodies in the chick, but not in the pigeon.

Vaccinia Virus Inclusions in Epidermal Cells of the Cornea of the Rat.—Scarification of the rat's cornea is followed by a well-marked lesion in the epidermal layer. Nucleolar extrusion is of common occurrence in the cells (figs. 22, 23 (*nx*)). The same occurs in the cornea of the sheep with sheep pox (figs. 25 to 27). These nucleolar extrusions are no doubt what have been called Guarnieri bodies. They are not virus bodies. The epidermal cells immediately surrounding the centre of the lesion contain clusters of granules which develop as shown in figs. 28 to 31. These are regarded as specific virus formations in the regenerating epidermal cells.

The epidermal cells throughout the area of the lesion contain vacuoles, inside the larger of which is a finely granular substance. These vacuoles originate in intimate relationship with the Golgi apparatus. What is regarded as the course of development of these inclusions is shown in figs. 32 to 41. Except for the absence of the strongly osmophil covering substance, they bear a striking resemblance to the virus bodies of the chick. These vacuolar inclusions with their finely granular contents are regarded as the specific virus bodies produced by vaccinia in the epidermal cells of the cornea.

Action of Vaccinia Virus on the Cells of Tumours and of the Testis.—No specific virus bodies were formed when vaccinia virus was injected into tumours, or into the testes of rats and mice. The centre of the inoculated area became necrotic after a few days. In the tumours the necrotic area was surrounded by cells with considerable vacuolation of their cytoplasm (figs. 42 to 45). Similar vacuolation was seen in the cells of the testis. The mitochondria were destroyed, the nuclei underwent chromolysis (figs. 49 and 50) and became pycnotic (fig. 47), but the Golgi apparatus often persisted till a relatively late stage of degeneration (figs. 46, 47 and 51).

(For general cytological conclusions see also § 7, p. 415.)

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DESCRIPTION OF PLATES 26–30.

PLATE 26.

FIGS. 1–8.—*Vaccinia virus bodies in epidermal cells of the skin of the chick.*

Figs. 1–5, successive stages in the development of the virus bodies drawn from unbleached Schridde preparations. Figs. 6–8, similar stages drawn from preparations bleached with hydrogen peroxide, previous to staining with iron-hæmatoxylin. (*m*, mitochondria; *vb*, virus body; *vv*, virus vacuole; v_1—v_7, successive stages in the development of virus bodies.)

PLATE 27.

FIGS. 9–21.—*Vaccinia virus bodies in epidermal cells of the chick.*

Figs. 9 and 13–16, show the relationship of the developing virus bodies (*vv*, *vb*) to the Golgi apparatus (GA) in epidermal cells of the skin. Figs. 10–12, show the same process in epidermal cells of the cornea. (Modified Kopsch method.)

Figs. 17–21, show the development of virus bodies in epidermal cells of the chick's cornea. (*m*, mitochondria; *vb*, virus body; *vv*, virus vacuole; v_1—v_5, successive stages in the development of the virus bodies.)

PLATE 28.

FIGS. 22–31.—*Virus bodies in the epidermal cells of the cornea of the sheep and rat.*

Figs. 22–24 and 28–31, drawn from the cornea of the rat scarified with vaccinia virus. Figs. 25–27, from the cornea of the sheep with sheep-pox.

Figs. 22 and 23 show nucleolar extrusion (*nx*) in the cells of the rat, and figs. 25–27, the same stype of nucleolar extrusion (GT, GS) in cells of the sheep cornea. Fig. 24 shows the vaccinia virus body (*g*) in the case of the rat.

Figs. 28–31, development of the granular structures, Schütz granules (G), which occur only in cells immediately surrounding the centre of the lesion in the epidermis.

PLATE 29.

FIGS. 32–41.—***Development of what, in epidermal cells of the rat cornea, is regarded as homologous with the virus bodies of the epidermal cells of the chick.***

Fig. 32, normal cell with net-like Golgi apparatus (GA). Fig. 33, spreading out of the apparatus (GA). Fig. 34, reversal in polarity of the apparatus (GA) and formation of vacuoles (*v*). Fig. 35, increase in number of vacuoles (*v*) and fragmentation of Golgi apparatus (GA). Fig. 36, coalescence of vacuoles (*v*) and appearance of a finely granular material (*g*) inside the largest vacuole. Figs. 37–40, increase in size of the vacuole with its contained substance, and the reduction of the cytoplasm to a mere peripheral layer surrounding the vacuole. Fig. 41, final stage of a cyst-like cavity containing the finely granular substance (*g*).

(Figs. 32–40 drawn from modified Kopsch preparations, fig. 41 from a Schridde preparation.)

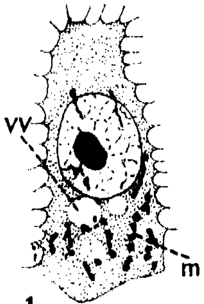
PLATE 30.

FIGS. 42–51.—*Cellular degeneration in a mouse carcinoma* (2146), *and in the rat testis resulting from injection of the vaccinia virus.*

Figs. 42–45, vacuolation of the cytoplasm of the tumour cells as the result of the toxic action of the injection.

Figs. 46–51, spermatocytes of the rat testis. Figs. 46–48 show the persistence of the Golgi apparatus (GA) during degeneration of the cell.

Figs. 49 and 50 show nuclear degeneration and disappearance of the mitochondria. Fig. 51 one of the masses of fused cells (plasmogamy) with remains of several nuclei, and Golgi fragments. Note the vacuolations of the cells of figs. 48 and 51. (*d*, vacuoles in the cytoplasm; GA, Golgi apparatus; *m*, mitochondria; *s*, sphere substance.)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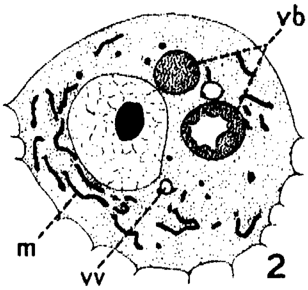
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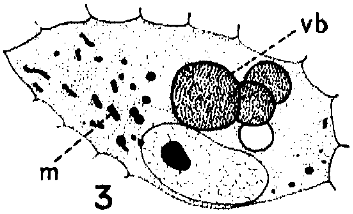
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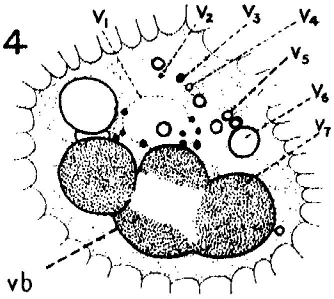
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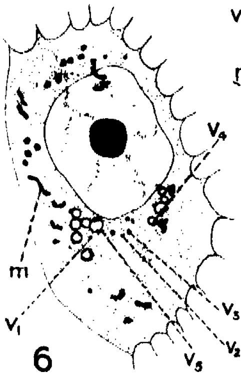
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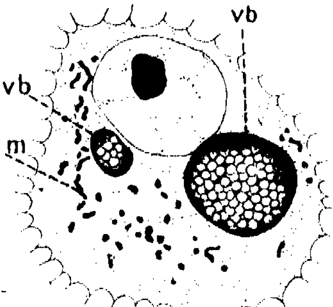
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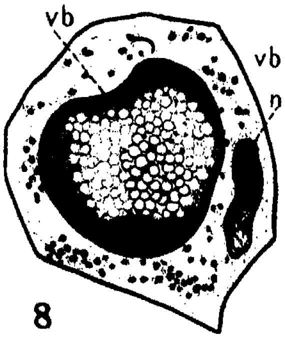
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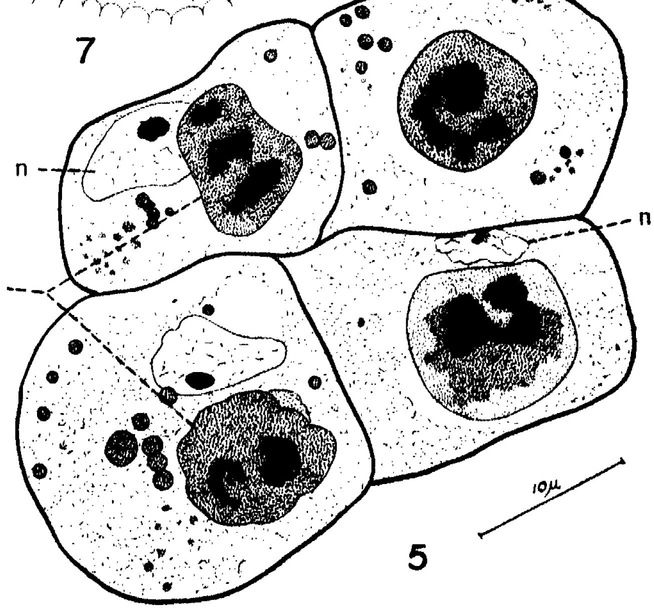
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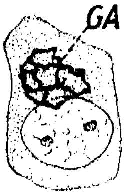

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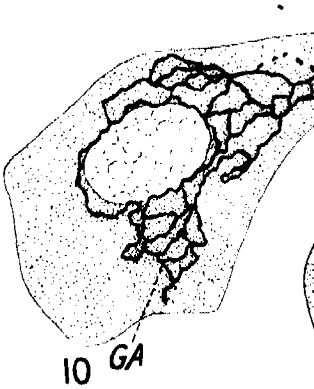

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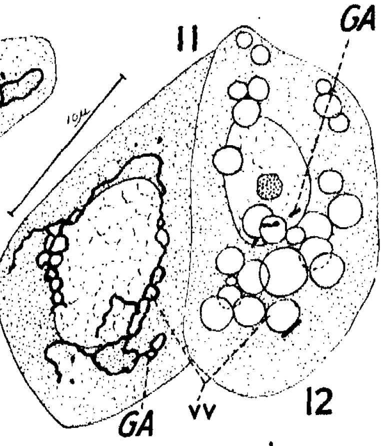

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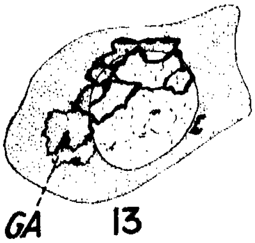

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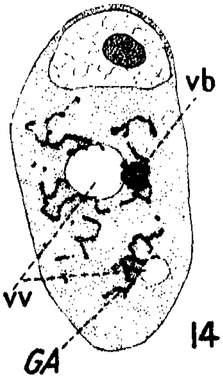

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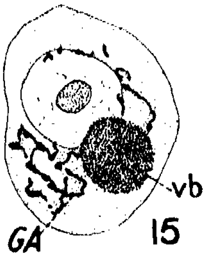

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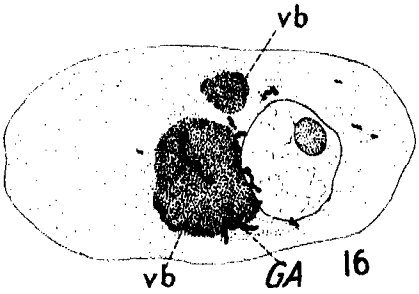

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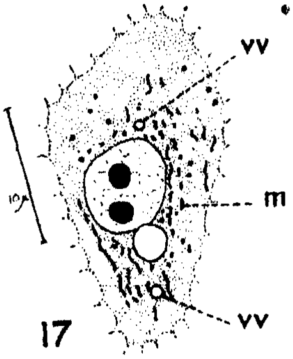

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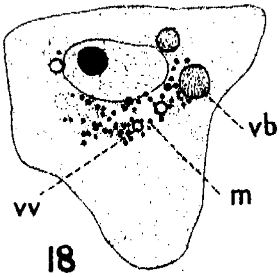

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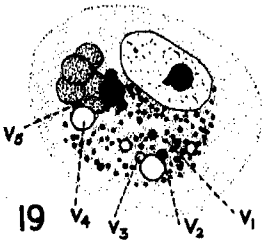

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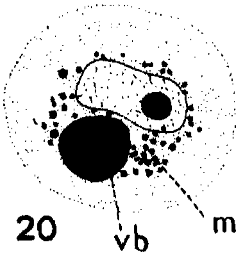

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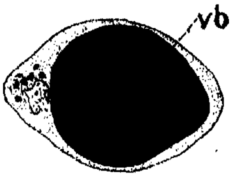

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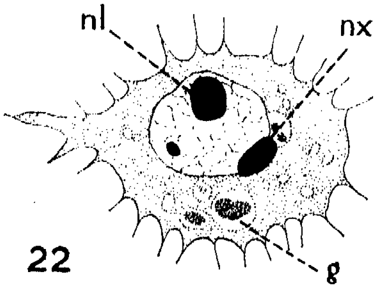

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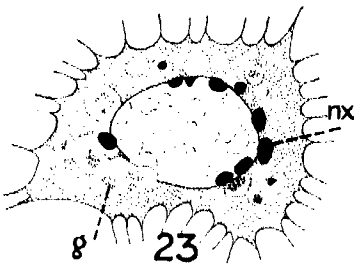

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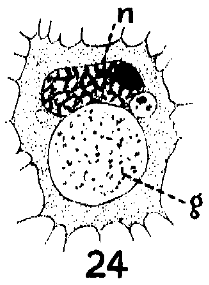

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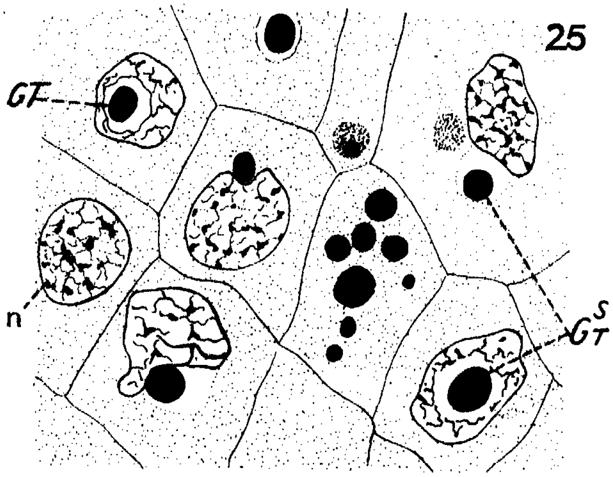

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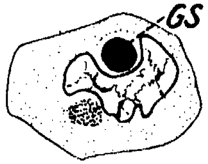

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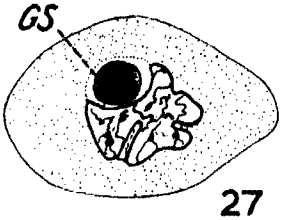

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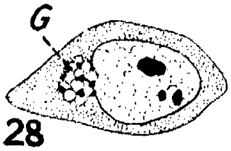

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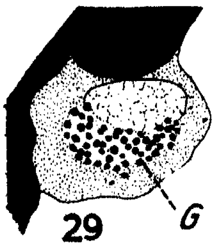

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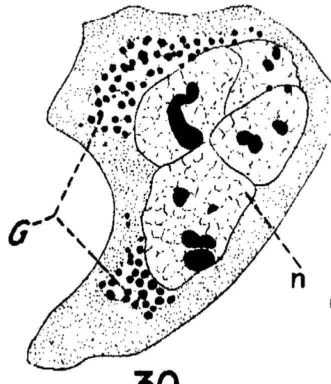

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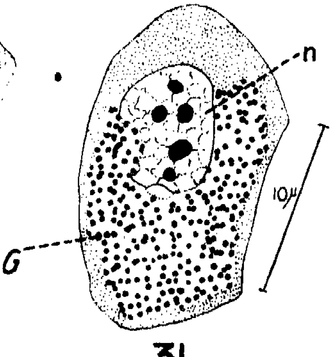

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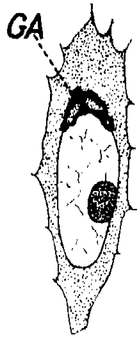

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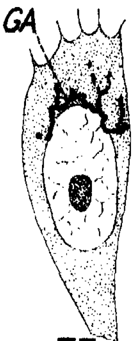

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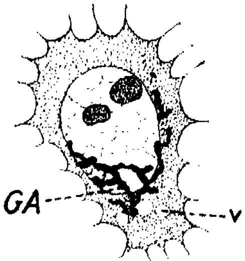

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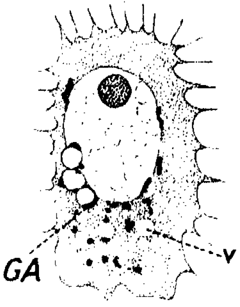

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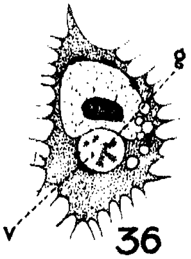

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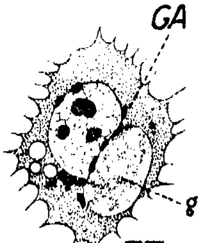

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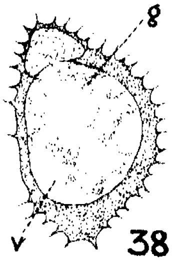

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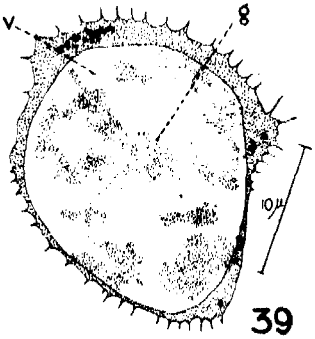

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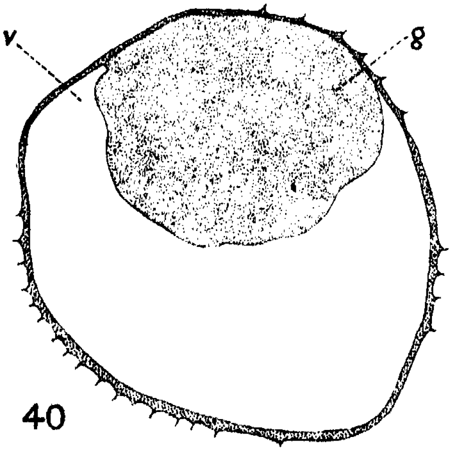

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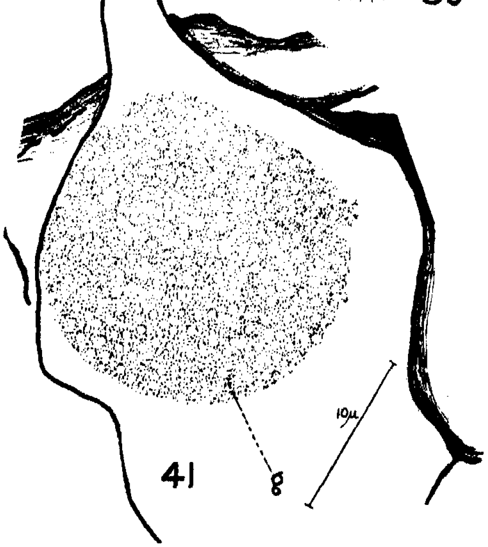

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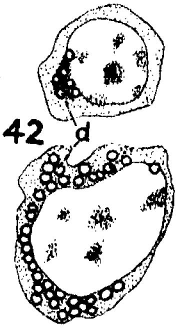

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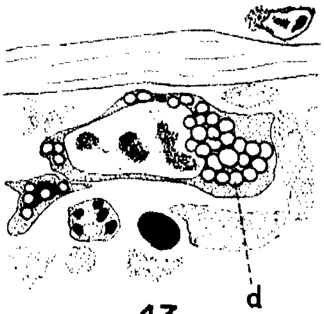

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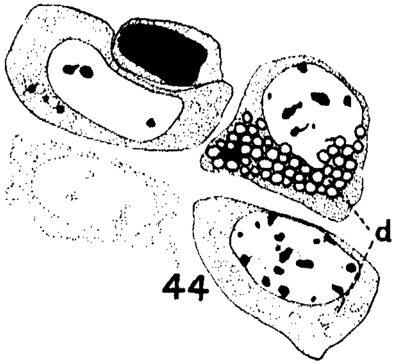

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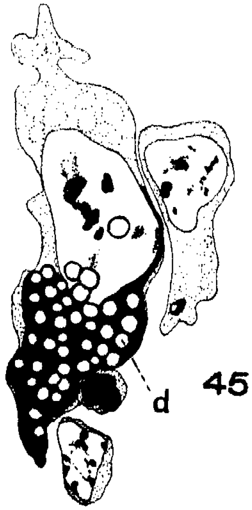

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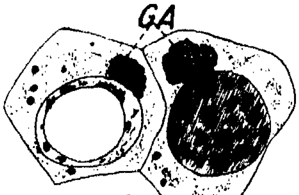

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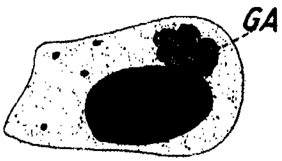

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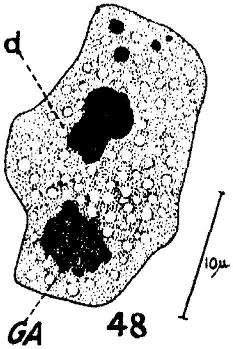

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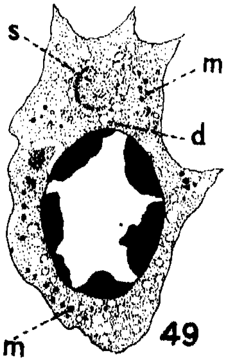

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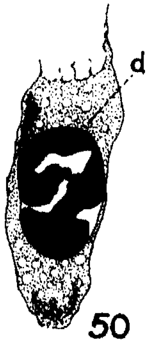

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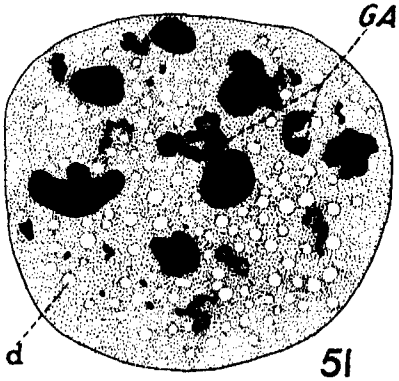


51

On the Chemical Evidences of the Organic Content of Human Enamel.

By Evelyn Sprawson, M.C., L.R.C.P. Lond., M.R.C.S., L.D.S. Eng., and
Frank W. Bury, M.Sc.Vict., A.I.C.

(Communicated by Prof. W. Bulloch, F.R.S.—Received December 10, 1927.)

Part 1.—Historical.

Although many analyses of enamel have been published, a comparison of these analyses shows that there are many discrepancies in their results, particularly in relation to the presence of organic matter. The principal chemical findings with reference to the presence of organic matter have been as follows :—

Von Bibra (1) in 1844 gave as the organic content of human enamel 3·59 per cent. in adult males and 5·97 per cent. in adult females. Most other analyses have been done on enamels derived from the lower animals, and though not exactly comparable to human enamels the results are appended, as being of interest.

Fremy (2) in hippopotamus enamel found 10 per cent. organic matter. Aeby (3) in the enamel of the cow found 3·60 per cent. organic matter. Hoppe-Seyler (4) in the horse found 4·74 per cent. organic matter, but in the dog he found 0 or ? 0 per cent. Until Tomes (5) published the results of his enquiry in 1896, no one seems to have taken into account the possibility that water might be a constituent of completed enamel.

Tomes, after making allowance for the presence of water in combination, came to the conclusion that there was no organic matter, "or not enough to amount to a weighable quantity," and confirmed this by a control, in which he used lime phosphate and carbonate in the proportions in which they exist in enamel, with the addition of 0·25 per cent. of dry collagen. In this mixture "the presence of the proteid was established with the utmost plainness," so he concluded that there was less than 0·25 per cent. of organic matter in enamel.

Tomes did, however, find that enamel, powdered and digested in weak baryta water for 24 hours, became cloudy on acidifying with acetic acid, which cloudiness, after being allowed to settle, was found to be too small in amount to weigh, and so demonstrated a trace of organic matter by qualitative means. He used the enamels of man, horses and elephants, and found that they showed no differences.

Bertz (6) in 1899 gave the organic content of the enamel of man as being 6·822 per cent., and that of the calf as being 16·56 per cent.

Lovatt Evans (7) in 1913 published an analysis of two samples of human enamel. These samples had first been washed in dilute hydrochloric acid, which might produce a source of error by dissolving out a small amount of acid soluble organic matter. He found that " organic matter is present and that its amount is from 1 to 2 per cent. at least," but continuing, makes the statement that " the relative amounts of carbon and nitrogen do not agree very well, the nitrogen being much higher than would correspond with the carbon found." He explains this by naming no less than three probable sources of error, and, further, states " great accuracy . . . is not claimed."

PART 2.—THE SCOPE OF THE PRESENT ENQUIRY AND THE OBTAINING OF MATERIAL.

Taking " organic " matter to mean compounds of carbon (other than carbonates) and nitrogen, such as are found in living tissues, our endeavour was to estimate the total carbon and nitrogen in enamel after the removal of carbon dioxide. Then *allowing for the whole of these two elements* in this residue to have been present in combination as some form of protein, one should find them present in the average approximate ratio (8) of carbon 3·3, nitrogen 1·0 (one of the analyses in this reference gives the ratio as carbon 3·55, nitrogen, 1·0, but the proportions vary in different forms of protein).

The difficulties and possible sources of error which had beset previous investigators in this subject seem to have been along four main lines :—

(i) The procuring of enamel entirely free from dentine or dentine *débris*.
(ii) The fact that water of crystallisation is present in combination with enamel.
(iii) The procuring of a sufficient quantity.
(iv) The fact that some constituents of enamel are more resistant to the action of certain acids than others, which has led to the presumption (histologically rather than chemically) that when there is an undissolved portion it is necessarily of an organic nature.

In addition to the above, further possible sources of error occurred to us :—

(v) The possible presence of Nasmyth's membrane on the enamel examined.

(vi) The fact that if dry teeth were used some foreign organic matter might have been drawn into the depth of any fissure or unevenness on the surface of the tooth.

(vii) The difficulty of avoiding adventitious contamination of samples in obtaining them.

(viii) Unavoidable contamination.

We have, we think, avoided these sources of error, except the last. This "unavoidable contamination" was due to particles of the abrasive material, used to separate the enamel from the teeth, becoming mixed with the samples. Steel files were used to obtain the samples, and we are informed by Dr. Walter Rosenhain, F.R.S., that carbon is present in such steel from 0·8 to 1·2 per cent. To remove this contamination as far as possible, each specimen was exposed and shaken about close to a powerful electro-magnet, such as is used for removing steel foreign bodies from the eye; most of the steel particles were removed in this way, but that traces remained became evident later on.

The teeth used were not specially selected; in most cases they were non-carious, but in all groups (not in all samples) some were slightly carious—such portions naturally were avoided when obtaining samples; some had slight opacities in their translucency, believed not to be due to incipient caries, and in many the enamel had not a very even surface. The teeth in all cases have been saved from which each sample was obtained, and are available for inspection if desired.

None of these teeth had ever been allowed to become dry; all, on extraction, had been placed immediately in 5 per cent. formic aldehyde and left in it till required. When they were required they were boiled in water, cleaned with a nail brush and cloth to remove any adherent soft material, and returned to water.

In order to remove Nasmyth's membrane or its remnants, and salivary calculus in the case of older teeth, each tooth was then cleaned on all aspects with a wet pumice brush on a lathe. This mechanical cleansing might obviously not remove organic particles of extraneous matter or of Nasmyth's membrane from deep fissures or crevices, neither would a file get into such places.

The teeth were then again washed to get rid of particles of pumice, and each, when taken from the water, was dipped momentarily into absolute alcohol to dry the outside, then clamped by the root portion in a small hand vice and filed with steel files, previously cleaned in ether, on to a slab of glass similarly cleaned. As much as one dared was filed from each tooth, the risk of going too deeply and on to the dentine being borne in mind. As each tooth was filed the surface was examined with a lens, to see that the dentine had not been reached, and the filings were transferred to ether-cleaned Petri dishes with covers. Several times one did go too deeply and had to discard the filings, ether-clean everything again, and start afresh on a different tooth.

Each sample was about 0·4 gram in weight. It took 10 premolars to give this weight of enamel, and several hours' work for each such sample. This was after one had obtained suitable teeth. In view of the fact that the enamel of recently erupted teeth is said (9) to be less highly calcified than it is later on in life, and that the enamel of deciduous teeth is believed (10) to have a greater organic content than the enamel of permanent teeth, we obtained our samples from three sources :—

1. The first group of samples was obtained from premolars which had been removed from children of from 12 to 14 years of age ; these represent *young human enamel.*
2. The next group was obtained from premolars removed from people of not less than 50 years of age ; these represent *adult human enamel.*
3. The last group was obtained from *deciduous molar teeth* of various ages.

It will readily be gathered that in each sample, particularly in the first group of unworn young premolars, the outermost portions of enamel, where its prismatic structure tends to be lost and becomes homogeneous, as recorded by Mummery (11), may have been in preponderance ; but we do not think it would be much so, and this would probably be counterbalanced by some deep portions from so near the dentine that "enamel spindles" and "tufts" were doubtless included. Twenty samples were prepared as follows :—

Young human enamel—samples 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 14, 15.
Adult human enamel—samples 12, 13, 17, 18, 19, 20.
Enamel from deciduous teeth—sample 16.

In sample 14, the enamel was taken from the sides of the crown only ; this was done in order that a more accurate comparison with adult human enamel might be made, as, owing to attrition, no filings could be taken from the occlusal surfaces of the teeth from which such samples were made. In samples Nos. 9 and 16 errors in technique were discovered during the tests, so they were discarded, and in samples Nos. 19 and 20, between preparation and testing, contamination with some foreign matter obvious to the naked eye was discovered, so these also were discarded.

PART 3.—CHEMICAL TESTS.

In every case, other than the qualitative estimation of nitrogen, controls were made in order to test the purity of the reagents used and the freedom of apparatus from contamination

I. *Estimation of Nitrogen. Qualitatively.*—0·6 gram of the enamel (samples 1 and part of 2, young human enamel) was fused with metallic sodium; this was dissolved in water and the resulting solution evaporated to dryness with a little ammonium sulphide; this was acidified with hydrochloric acid and a solution of ferric chloride added. No red coloration was produced. This indicates that the percentage of nitrogen must be below 0·1 per cent., equivalent to 0·638 per cent. of protein.

II. *Estimation of Nitrogen*, by Kjeldahl's method (samples 3 and 4, young human enamel).—If the proportion of nitrogen present is in excess of 0·04 per cent., this method would indicate it.

The samples of enamel were heated with concentrated sulphuric acid containing potassium sulphate. This solution was neutralised with sodium hydrate and excess of sodium hydrate added. The resulting solution was distilled and the distillate collected in 20 c.c. of 0·1 N hydrochloric acid. No acid was found to be neutralised.

Considering the quantity of enamel taken and the degree of accuracy possible with the apparatus used, the percentage of nitrogen present must therefore be less than 0·04 per cent., corresponding to an organic content of less than 0·25 per cent.

III. *Estimation of Nitrogen. Quantitatively.*—To estimate quantitatively the amount of nitrogen in the enamel a more sensitive method was therefore essential. The method as in II was adopted, but the ammonia content of the distillate was estimated colorimetrically by means of Nessler's solution.

The delicacy of this test is such that it will indicate the presence of 0·000005 gram of nitrogen, which, considering the weight of each sample to be about 0·4 gram, gives a sensitivity of 1 per 80,000, or a percentage of 0·00125 of nitrogen.

The results are appended :—

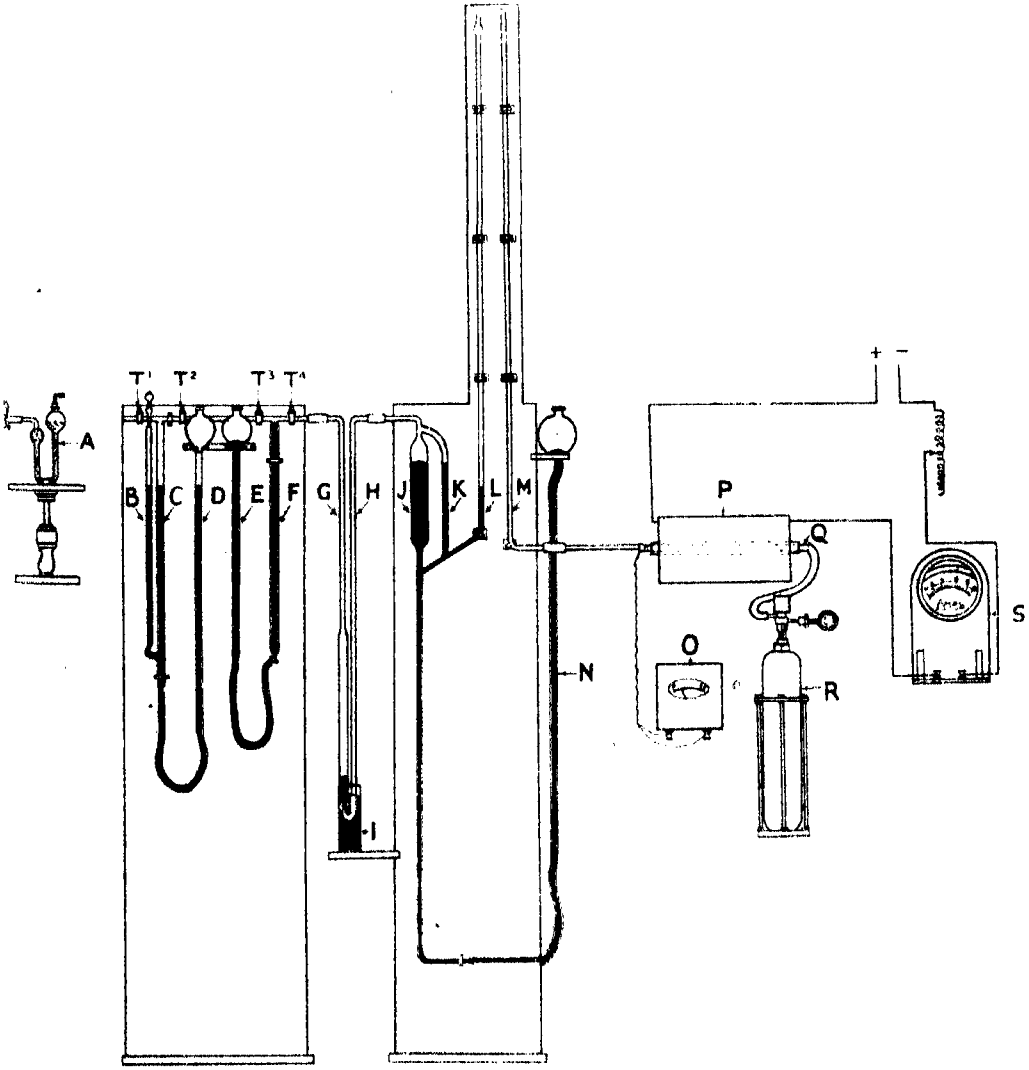
Number and nature of sample.	Weight of sample.	Percentage of NH_3.	Percentage of N.	Percentage of protein (factor 6·38).
	gram.			
5 and part of 2, young enamel	0·8288	0·0266	0·022	0·14
8, ditto	0·3024	0·028	0·023	0·15
12 and 13, adult enamel	0·8092	0·027	0·022	0·14
14, young enamel from sides of crown only	0·4529	0·026	0·02	0·14
16, deciduous teeth	0·3333	0·028	0·023	0·15
17, adult enamel	0·3968	0·028	0·023	0·15
18, ditto	0·3578	0·028	0·023	0·15

IV. *Estimation of Carbon. Quantitatively.*—The enamel samples were flamed in a glass dish, dissolved in hydrochloric acid, and the solution evaporated to dryness on a water-bath. To the residue, about 5 c.c. of strong hydrochloric acid was added and the solution again evaporated to dryness.

Thus all carbon present as carbonate was removed. In order to detect the presence of carbon in the dried residue an apparatus was used which had been designed by Dr. W. H. Patterson, of the East London College, for work of a similar nature. Dr. Patterson placed his apparatus and skill in its use at our disposal, for which we make grateful acknowledgment.

The apparatus (see figure) consists of a furnace P heated electrically to 1000° C., a Sprengel pump JKLM, and an apparatus for measuring the evolved gases BCDEF. The residue was placed in P and oxygen passed through the apparatus, the oxygen escaping at I. Sufficient oxygen was then withdrawn by lowering N to reduce the pressure to about 380 mm. of mercury, as shown by the rise of the mercury in tube H. The tube H was then placed under the collecting tube G, and the temperature raised to about 1000° C. The evolved gas was transferred to the measuring tube C. This was done by altering the level of the tubes D and E and by the use of the taps T_4, T_3 and T_2. A little water was passed into C to remove any hydrochloric acid gas. The carbon dioxide was measured by absorption in the potash bulb A. *In all cases* a small amount of ferric chloride was found in tube Q, thus demonstrating the presence of "unavoidable contamination." The results are appended :—

Number and nature of sample.	Weight of sample.	Volume of CO_2.	Percentage of carbon.	Percentage of protein (factor 1·88).
	gram.	c.c.		
6, young human enamel	0·4335	0·87	0·098	0·18
7, ditto	0·3978	0·85	0·11	0·21
10 and 11, ditto	0·8400	1·73	0·11	0·21



Conclusions.

1. Human enamel contains a small quantity of organic matter.

2. The maximum amount of protein present calculated from the highest amount of the nitrogen content found is therefore 0·15 per cent.

3. The maximum amount of protein found calculated from the highest amount of the carbon estimation is therefore 0·21 per cent., but the presence of free iron-giving carbon as " unavoidable contamination " must here be taken into account.

4. The remarkable fact emerges that the organic content of the samples appears to be independent of the age of the teeth or the dentition (deciduous or permanent) from which the samples were obtained.

5. The high carbon : nitrogen ratio of 0·10 : 0·027, *i.e.*, 3·7 instead of 3·3, the usually accepted figure for protein, may be accounted for by the fact that the samples used for the estimation of carbon were not entirely free from iron, which contains about 1 per cent. carbon in combination.

These findings, then, confirm, but with tests of greater delicacy and accuracy and by quantitative means, the work of Tomes in 1896. Tomes wrote, " I find none, or not enough to amount to a weighable quantity." He did, however, find a trace (qualitatively), " less than 0·25 per cent."

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A Quantitative Study of the Course of Fungal Invasion of the Apple Fruit and its Bearing on the Nature of Disease Resistance.—Part I. *A Statistical Method of Studying Fungal Invasion.*

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(Communicated by Prof. V. H. Blackman, F.R.S.—Received March 31, 1927.)

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CONTENTS.

		Page
I.	Introduction	427
II.	The Material used	428
III.	The Experimental Method	429
IV.	A Method of Treating the Primary Data for Statistical Purposes	429
V.	The Distribution of Radial Advance and Percentage Weight of Rotted Tissue in Random Samples of Apples	434
VI.	Evidence for the Specific Nature of Resistance to Fungal Invasion	439
VII.	Summary	442

I.—*Introduction.*

In the course of investigations on the incidence of disease in apples stored at low temperatures, which have been in progress since 1920, it soon became evident that a regular series of fungal species appeared successively during the period of storage. It seemed desirable to investigate the problem more closely with a view to disentangling the factors concerned in the succession. The first method employed for the purpose consisted in taking a periodical census of the population of apples and noting the number of apples "invaded" and "not invaded." The need for improvement on this method of studying fungal invasion became clear in the course of the investigation, and led to the development of the technique, described in the present paper. The method of the periodical census has been utilised by M. N. Kidd (1) who has constructed curves of the progress of fungal invasion and from them has derived "mortality curves." The interpretation of these "mortality curves" is, however, somewhat difficult, representing, as they do, not only the effect of varying resistance in the apple, but also the interaction of this factor with the chances of casual infection, which includes the varying powers of penetration possessed by different species of fungi. The distribution of the two classes of apples "invaded" and "not invaded," depends on the interaction of three factors:

(1) the conditions of infection. (2) the progress of invasion after infection, and (3) the final stage of invasion. The bearing of these factors on the problem of invasion of apples will now be briefly considered.

(1) Unless precautions are taken to ensure equal chances of infection by utilising a sound technique of inoculation the problem of the incidence of disease in stored apples is confused by factors such as the unequal distribution of infecting organisms in individual apples of the population, the unequal distribution of spores on individual apples in relation to the distribution of the lenticels or other points of entry, and so on. The method employed in the course of this investigation has already been described (2) and has proved itself adequate.

(2) It is the problem of the progress of disease after infection which will be mainly dealt with, and the method of study will be fully described subsequently in this paper. It is clear, however, that the method previously employed takes no account of the degree of infection, and indeed the assessment of the virulence of two species or strains of fungi will depend on the time at which the census is taken, and should the onset of the invasion be much delayed after the entry of the fungus the main progress of the invasion may be altogether missed.

(3) In the course of the investigation it became clear that after the primary infection the further development of the fungus may be arrested for a long period of time although the fungus remains viable. Such arrest of invasion, which has been called "interference" in this paper, was previously noticed in studying the "spotting" of apples (3) and has been met with on several occasions in the course of this work. This "interference" must be taken into account in assessing the virulence of particular species or strains. The phenomenon is always confined to a varying proportion of the total number of apples in which rotting is checked in the early stages of invasion, while in the remainder invasion proceeds normally to completion. Unless the course of the invasion is followed in such cases an entirely erroneous conclusion will be reached, since by mere inspection apples showing "interference" would be placed in the class "not invaded."

II.—*The Material Used.*

The varieties of apples used throughout this investigation were Cox's Orange Pippin from Burwell, Cambridgeshire and Bramley's Seedling from six localities.

The fungi principally used were originally isolated from apples as previously described (3, 4) and consisted of *Botrytis* sp., *Cytosporina ludibunda*, *Fusarium* sp. (5), *Pleospora pomorum* and *Polyopeus aureus*.

III.—*The Experimental Method.*

At the time of gathering, apples were selected as far as possible of uniform size and free from obvious blemishes. They were then infected at two opposite equatorial points by the standard method already described (2) and transferred as soon as possible to the Low Temperature Station, Cambridge, where they were kept under constant conditions of temperature and humidity. For this work two temperatures were mainly employed, viz., 3° C. and 12° C. From time to time batches of inoculated apples were despatched from Cambridge to London for examination. The apples were then individually weighed and the superficial diseased areas on the two sides were either measured, or the diseased tissue removed and weighed. The measurements taken of the two diseased areas were the mean diameters, obtained by averaging the lengths of two diametral arcs at right angles. The weight of the diseased tissue was obtained by carefully scooping out the decayed areas on each side in turn and weighing the apple after each operation. When invasion was nearly complete it was not possible to record the weights in the two sides separately.

In cases where "interference" had occurred it was found that the rotten tissue had dried out and hollow spaces were left inside the apple. In order to estimate the extent of the invasion prior to drying in such cases, the volume of the hollow was measured by filling with water from a burette and so obtaining the volume. The specific gravity of the apple tissue was then determined and these volumes were converted into weights.

In all doubtful cases where contamination was suspected the causal organism was re-isolated, and unless the original infecting fungus was recovered unassociated with others, the apple was rejected.

The results here recorded are based on a selection from experimental data involving 15,000 separate inoculations, which were carried through by competent helpers.* The main body of results will be dealt with in a second paper (Part II).

IV.—*A Method of Treating the Primary Data for Statistical Purposes.*

The kinds of problems which will be dealt with in the second part of this paper are as follows:—

(1) Comparison of the infecting power of different species and strains of fungi in apples of a given variety.

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* The authors' thanks are due to Mrs. Cartwright, Mrs. Harvey and Mrs. Horne for the skilful and efficient way in which this work was done.

(2) The resistance to invasion of different varieties of apples to certain fungal species and strains.

(3) The change in resistance undergone by apples during storage and during the course of invasion by fungi.

The infecting power of the fungi must be assessed from the amount of damage done in a given time, or the time taken to do equivalent amounts of damage. Three possible measures of the damage done will be considered: (*a*) the superficial area of discoloration; (*b*) the weight of rotten tissue produced in a given time, either as absolute weight or as percentage of the weight of the apple; (*c*) a measure derived from the weight of rotten tissue, which will be called "radial advance."

The method of assessing the amount of damage by the measurement of superficial areas was used for one experiment only, before the weighing method had been tried. It was abandoned, since it was found that the area of discoloration apparent on the surface was in many cases smaller than the real affected area, a thin superficial layer of sound tissue being left just below the skin.

The weight of rotten tissue may be accurately determined, but the following considerations indicate, however, that the weight of rotten tissue is not an ideal measure to employ.

It has been assumed that infection proceeded from two points at the opposite ends of a diameter in the equatorial plane of the apple, and that the advance has followed spherical shells centred at the points of infection. The further assumption has been made that the apple is spherical in form and is uniform throughout. If the apple were a sphere of unlimited size and invasion proceeded at a uniform rate then the mass of decayed tissue would increase as the cube of time.

Actually the apple approximates to a sphere of very limited size compared with the infected volume and hence only in the very early stages would this relation hold. The curve of total mass of decayed tissue plotted against time is in fact of complex sigmoid form. It can be shown by geometry that in a sphere of radius R, when the front of the invaded zone has reached a certain distance from the point of inoculation, where x is the distance divided by the length of the radius of the sphere, the volume (V) of affected tissue may be expressed by the equation

$$V = \frac{\pi}{12} R^3 x^3 [8 - 3x],$$

when $x = 2$ this becomes $V = \frac{4}{3}\pi R^3$ and is the total volume of the sphere. The distance from the point of inoculation to the front of the invasion, expressed as a fraction of the radius, will subsequently be called "radial advance." Taking the total volume of the sphere as 1, the proportion (v) of the total volume affected will be given by

$$v = \frac{x^3 (8 - 3x)}{16}.$$

This relation holds only when invasion proceeds from one point on the surface of the apple. When two points of infection are considered, the proportion of the total volume affected will be double the amount calculated from the formula in the early stages, until the advancing fronts of invasion meet at the centre of the sphere, when 63 per cent. of the total volume has been invaded. After

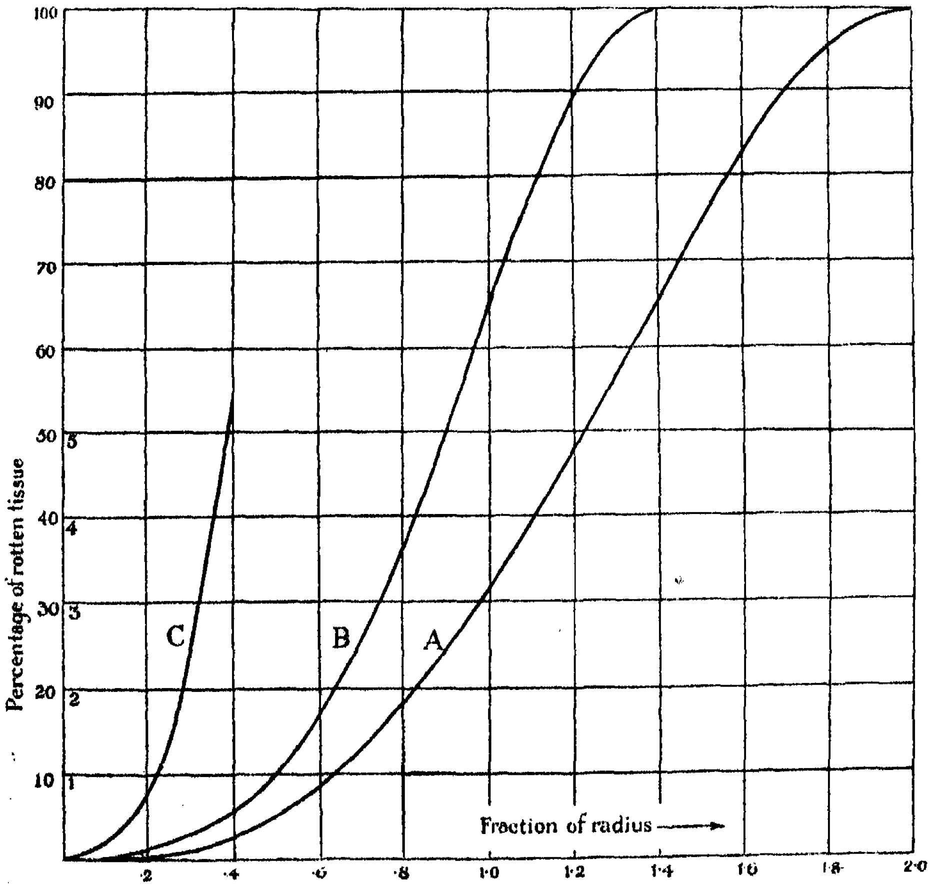

FIG. 1.—Curve showing the relation between the percentage of rotted tissue and radial advance, expressed as a fraction of the radius. A, for apples inoculated at one point. B, for apples inoculated at two points. C, early part of curve B enlarged 10 times.

this, overlapping will occur and a correction has to be made for the volume of overlap, as proportion of total volume of sphere, given by the expression

$$v' = \frac{(x-1)(4x^2-2x-2)}{4}.$$

Fig. 1 shows the curve of percentage volume of mass rotted (v) plotted against x (x = radial advance) for a sphere inoculated at one and two points respectively, and in Table I the values are given of the percentage mass (V) rotted for increasing radial advances. It is evident that complete rot corresponds to a penetration of 1·4 ($= \sqrt{2}$) times the radius.

Table I.—Relation between Radial Advance and Percentage of Apple rotted for one and two Points of Inoculation.

x = Radial advance.	V = Percentage of apple rotted.	
	1 point of inoculation.	2 points of inoculation.
	Per cent.	Per cent.
0·1	0·048	0·096
0·2	0·37	0·74
0·3	1·20	2·40
0·4	2·72	5·44
0·5	5·08	10·16
0·6	8·37	16·74
0·7	12·65	25·30
0·8	17·92	35·84
0·9	24·15	48·30
1·0	31·50	63·00
1·1	39·1	76·6
1·2	47·5	87·2
1·3	56·3	96·4
1·4	65·2	100
1·5	73·8	
1·6	82·4	
1·7	89·1	
1·8	94·8	
1·9	98·6	
2·0	100	

If the conditions of invasion approximate to those assumed in developing the equations given above, it is clear from the figures in Table I that equal increments in radial advance do not represent equal increments in percentage mass of rotten tissue. Hence if invasion is proceeding at a uniform rate the relation of percentage amount of rot, hence also the weight of rotten tissue, produced in two equal increments of time will depend on the particular stage of invasion at which the comparisons are made. Further the relations of amounts of rotten tissue will depend on the size of the apples inoculated, and if relative virulence is estimated by comparison of weights of tissue rotted, errors will be introduced unless a correction for size of apples is made. An example of the magnitude of the errors liable to be introduced by the effect of size of apple is shown in Table II.

Table II.—Errors introduced into Comparison of Invasion by Effect of Size of Apple.

Radial advance in cm.	Percentage weight of rot.		Ratio.
	Cox's Orange Pippin.	Bramley's Seedling.	
cm.	Per cent.	Per cent.	
0·5	0·656	0·254	2·58
1·0	4·88	1·92	2·54
1·5	15·05	6·10	2·47
2·0	32·38	13·58	2·38
2·5	56·84	24·78	2·29
3·0	87·14	39·80	2·19
3·5	98·62	58·40	1·69
	Weight of rot.		Percentage difference.
cm.	cm.	cm.	Per cent.
0·5	0·115	0·119	3·4
1·0	0·858	0·896	4·4
1·5	2·645	2·846	7·6
2·0	5·691	6·336	11·3
2·5	9·990	11·561	15·7
3·0	15·32	18·57	21·2
3·5	17·33	27·25	57·2

Mean radius Cox's Orange Pippin, 2·6 cm.; Bramley's Seedling, 3·6 cm.

Two varieties, Cox's Orange Pippin and Bramley's Seedling, are being compared. The mean radii for these varieties are Cox's Orange Pippin 2·6 cm., Bramley's Seedlings 3·6 cm. In the table are presented relative absolute and percentage weights of rotten tissue for these two varieties of apples invaded by a fungus assumed to be equally active in both varieties, when radial advance has proceeded for distances given in the first column of the table. In this hypothetical case the fungus is known to be equally virulent in either variety, yet by comparing either the weights of rotten tissue or the percentage weights a wrong estimate of virulence would result, and the errors involved would depend on the stage of invasion at which comparison was made.

This effect of absolute size of apples invalidates the use of absolute or percentage weights of rotten tissue in comparing the virulence of a given species of fungus in apples of the same or different varieties if these differ in absolute size. An ideal measure of invasion should be independent of the size of the apple, and moreover should give equal numerical values for equal intervals of time, if the rate of invasion remains constant. In so far as the assumptions made in deriving the radial advance from the percentage weight of apple affected are justified, radial advance will successfully eliminate the effect of absolute size, and will give equal numerical values for equal intervals of time if the rate of invasion of the fungus is constant. The primary data derived from weight of rotten tissue can therefore by conversion to radial advance be used to compare the course of invasion by fungi even where the rate of invasion differs in two cases, or apples of different sizes are used. Further, any change in resistance will be reflected in a rise or fall in magnitude of the rate of radial advance, and in no other way could changes in resistance during invasion be followed.

The method of dealing with samples which was finally adopted was as follows. The sum of the weights of rotted tissue recorded from the two sides was first converted into percentage of the total weight of the apple. From the curve in fig. 1 the percentage data were converted into percentages of radial advance. From the weights of the individual apples and the specific gravity characteristic for the variety of apple, the mean radius in centimetres of the apples constituting the sample was calculated, and this figure multiplied by the mean radial advance measured the penetration in centimetres. In this way apples of different sizes could be compared and the rate of invasion calculated over different periods of time and with different fungal species. The probable error of the figures obtained from each operation was calculated, and hence the probable errors of the final figures for the rate of invasion were known.

V.—*The Distribution of Radial Advance and Percentage Weight of Rotted Tissue in Random Samples of Apples.*

In practice the progress of invasion in a uniform set of apples by a given species of fungus is followed by taking samples at different times and determining the amount of rotten tissue in each apple by the method of weighing already indicated. Although the apples are originally selected as uniform as possible, yet in any one sample large variations in weight of rotten tissue from the individual apples are invariably found. These differences in the amount affected may be attributed to variations in resistance to invasion in individual apples. Considering two apples of different resistance inoculated with the same fungus, it may be assumed that the radial advance will be inversely proportional to the resistances of the two apples.* From the distribution of radial advance in a set of apples some information may be derived as to variation in resistance in individual apples in a sample. Since the resistance cannot be directly measured, its nature being unknown, and its magnitude being assumed to be inversely proportional to rate of radial advance, any conclusions as to resistance will depend for their value on the validity of the assumptions made in deriving radial advance from the experimental data of weights of rotten tissue. It may be well therefore to present evidence that the distributions of weights of rotten tissue in samples of apples of varying resistance actually conform with those expected if the conditions of invasion approximate to those postulated in the geometrical treatment of the invasion of a uniform sphere.

In fig. 2 is presented a set of distribution curves embodying the data from five

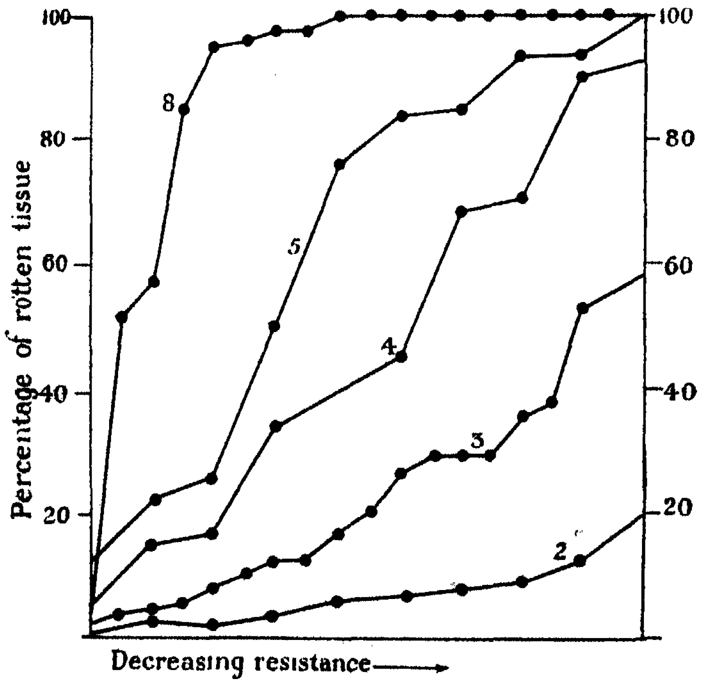

FIG. 2.—Distribution curves of percentages of weight of tissue rotted in samples of apples arranged in serial order of increasing weight, for comparison with the theoretical distributions in fig. 3.

2 = Cox's Orange Pippin 12° C., 1924–25. *Fusarium* strain B11.
3 = Bramley's Seedling, 12° C., 1924–25. *Fusarium* strain C21.
4 = Cox's Orange Pippin, 12° C., 1924–25. *Fusarium* strain A.
5 = Cox's Orange Pippin, 12° C., 1924–25. *Fusarium* strain C1.
8 = Cox's Orange Pippin, 12° C., 1925–26. *Botrytis* sp.

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* This will only be true up to the limit at which the fungus is growing at the maximum rate conditioned by internal factors.

selected experiments. The ordinates are the percentage weights of rot from each individual apple in the sample, which have been arranged in serial order along the abscissa. The various curves indicate the distribution of percentage weight of rotten tissue in individual apples for samples in which the invasion has proceeded to varying extents towards completion. These curves may be compared with those in fig. 3 which are purely theoretical and were constructed in the following manner :—

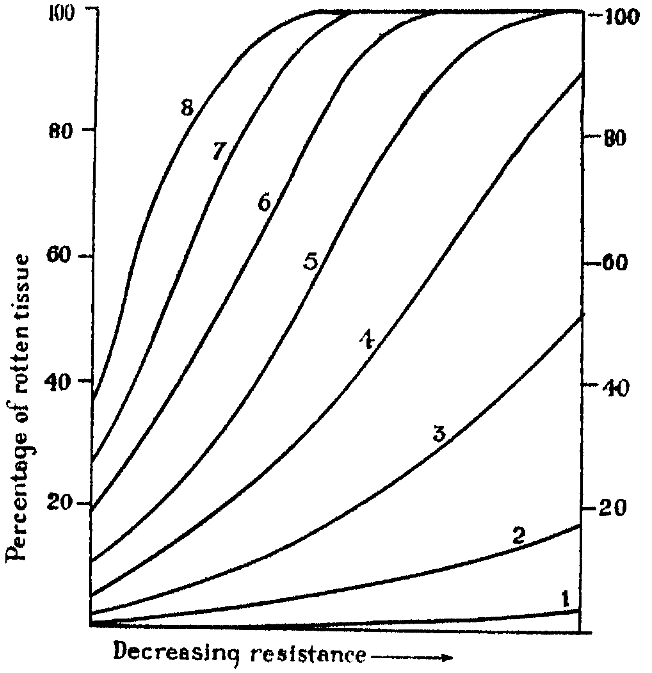

FIG. 3.—Theoretical distribution curves of percentage weight of tissue rotted, arranged in serial order, for apples of varying resistance after successive equal intervals of time (1–8).

Five hypothetical apples were considered characterised by rates of radial advance, for an assumed fungus, 0·3, 0·25, 0·20, 0·15 and 0·10 of the radius per unit of time respectively. The reciprocals of these values would give the relative magnitudes of the resistances of these hypothetical apples. The apples have been arranged in serial order of increasing radial advance, the apple of highest resistance being on the left of the diagram, the others being placed in order of decreasing resistance. Taking times 1, 2, 3, 4, etc., the radial advances after successive periods of time were found, and from the curve in fig. 1 these values of radial advance were converted into the corresponding percentages of rotten tissue.

It is the values of percentage of apple rotted which are presented as curves in fig. 3, each curve being marked with the period of time elapsed. The curves thus represent distribution curves of weight of rotted tissue, arranged in serial order for a given set of apples, after the lapse of varying periods of time; or they may represent equally well distribution curves which would be obtained after a given period of time from sets of apples, all of the same kind, but infected with fungi of different virulence. The resemblance of the theoretical and experimental distribution curves* lends some probability to the validity of the simplifying assumptions introduced into the geometrical considerations of the problem of invasion. Frequency distributions of percentage weight of rotten tissue and of radial advance in a large population of apples are shown in figs. 4 and 5.

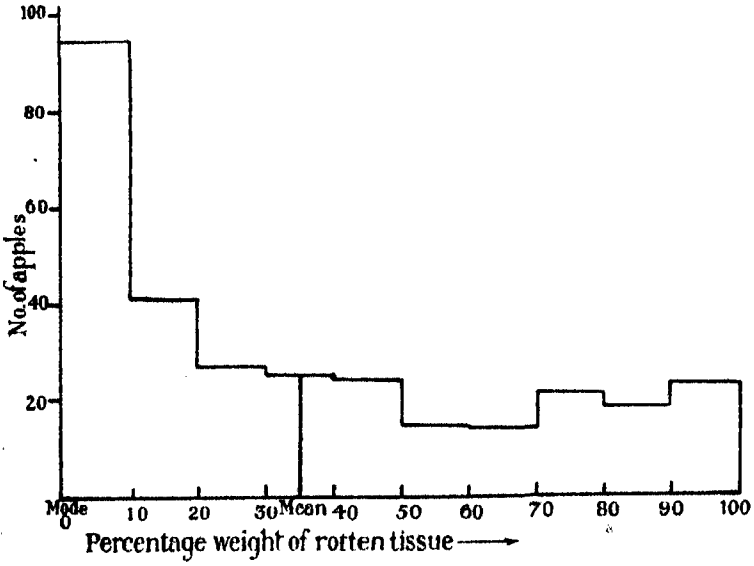

FIG. 4.—Frequency distribution of invasion in a set of 301 inoculated apples, measured in terms of percentage weight of rotted tissue.

Cox's Orange Pippin { 3° C., 1925–26; 12° C., 1925–26 } *Botrytis*.
" " { 3° C., 1925–26; 12° C., 1926–27 } *Cytosporina*.
Bramley's Seedling { 12° C., 1926–27; 20° C., 1926–27; 20° C., 1927–28 } *Cytosporina*.

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* A rigid test of the similarity of the curves in figs. 2 and 3 would require an independent measure of the resistance in individual apples. The only assumption which has been made is that resistance does vary in individual apples, and that the individuals in a sample can be arranged in a serial order of increasing resistance.

The data were derived from 301 apples in five samples, three sets of Cox's Orange Pippins (177 apples), and two sets of Bramley's Seedlings (124 apples).

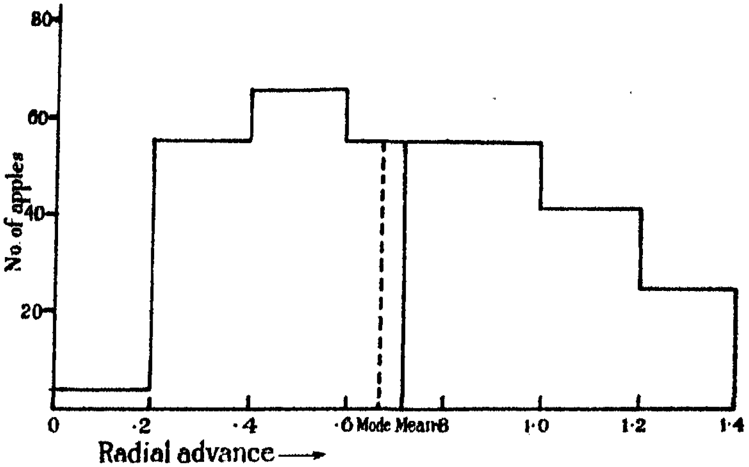

Fig. 5.—Frequency distribution of invasion in the same set of 301 inoculated apples measured in terms of radial advance.

The figures for percentage weight of rotten tissue were divided into 10 classes each covering a range of 10 per cent., and a frequency distribution was made of the number of apples falling into each class. This is presented in fig. 4. The percentage weights were then converted into radial advance, and the figures thus obtained divided into seven classes, each covering a range of 0·2 radial advance. The numbers of apples in each class are shown in fig. 5.

The distribution of percentage weights is seen to be highly asymmetrical, while radial advance distribution is only moderately asymmetrical. The standard errors of the means of the two distributions are 5·02 per cent. for percentage weights and 2·23 per cent for radial advance respectively; hence mean radial advance of a sample may be estimated with twice the accuracy of percentage weight of rotten tissue. In addition, therefore, to the advantage which radial advance has over weight of rotten tissue as a measure of invasion in eliminating the effect of size of apple and the stage of invasion reached, the added advantage of greater accuracy is obtained, so that samples of one-half the size may be used without loss of efficiency. Since distributions departing largely from symmetry are statistically inconvenient. the treatment of the results of the experiments to be reported in Part II of this paper would seem to require the choice of radial advance as the working variate.

The distribution of radial advance itself, however, tends to become increasingly asymmetrical as invasion proceeds towards completion. Increasing numbers of apples will fall into the class 100 per cent. rotten (radial advance 1·4), and the mode of the distribution will shift gradually towards the highest class, in which the majority of apples are finally found, simply because invasion can advance no further. It is evident then that for purposes of comparing the effect of given species of fungi on different varieties of apple, or comparing the virulence of fungal species or strains on the same variety of apple, the sample should be taken not later than the time when one of the series first shows a completely rotten apple.

From the foregoing considerations it is evident that radial advance provides a convenient scale in which to measure the individual resistance of the apple to fungal invasion, and defined in this way resistance is seen to be distributed in a nearly normal manner in a population of apples.

VI.—*Evidence for the Specific Nature of Resistance to Fungal Invasion.*

At this point it may be well to give evidence for the specific nature of variations in resistance in a population of apples. If the resistance within a population of apples varies, it might be expected that difference in resistance within an individual apple would be less than in any two apples taken at random. This has been confirmed by estimating the correlations between radial advance of a given fungus on two sides of individual apples as well as the correlations between weights of rotten tissue derived from the two sides. The data are presented below (Table III). Two varieties of apple have been used at two different temperatures, tested with three fungal species. The correlations for percentage weight of rotten tissue are enclosed in brackets. The correlation coefficients for radial advance are all positive and significant, and are in every case greater than the corresponding values for percentage weight, reflecting the smaller standard deviation of radial advance.

The correlations provide clear evidence of the specific value of resistance in individual apples.

Although the correlations so far presented indicate that the resistance at one pole of an apple tends to be similar to that at the opposite pole, examination of a set of data giving individual weights of the rotten tissue from the two sides reveals striking differences. It seemed possible therefore that the resistance of an individual apple might vary greatly from point to point around the circumference.

To test this point a set of 20 Bramley's Seedling apples was taken and each apple was inoculated at four points equidistant around the circumference. The results as determined in terms of radial advance after storage for 17 days are given in Table IV.

Table III.—Correlation between Estimates of Invasion on two Sides of Individual Apples.

Season.	Fungus.	Temperature.	Variety of apple.	Correlation coefficient (*r*).	No. of apples.
		° C.			
1925–26	*Fusarium.* Strain D	12	Bramley's Seedling	(+0·603) +0·874	19
	Botrytis. Sample I	3	Cox's Orange Pippin	(+0·479) +0.598	20
	Botrytis. Sample II	3	Cox's Orange Pippin	(+0·597) +0·655	16
	Cytosporina. Sample II	3	Cox's Orange Pippin	(+0·428) +0·657	16
	Cytosporina. Sample I	3	Cox's Orange Pippin	(+0·610) +0·662	20
1924–25	*Cytosporina*	3	Bramley's Seedling	(+0·556) +0·717	25
			Mean	(+0·546) +0·694	116

Table IV.—Radial Advance at four equidistant Points in Individual Apples Inoculated with same Fungus.

	a.	*b.*	*c.*	*d.*
1	0·26	0·25	0·22	0·28
2	0·28	0·24	0·24	0·27
3	0·41	0·30	0·37	0·38
4	0·37	0·27	0·28	0·23
5	0·41	0·28	0·26	0·27
6	0·43	0·28	0·28	0·29
7	0·39	0·26	0·27	0·30
8	0·37	0·22	0·36	0·24
9	0·44	0·18	0·38	0·33
10	0·47	0·28	0·38	0·28
11	0·54	0·40	0·48	0·29
12	0·38	0·30	0·32	0·28
13	0·46	0·33	0·36	0·32
14	0·50	0·28	0·36	0·28
15	0·46	0·28	0·40	0·36
16	0·60	0·56	0·45	0·40
17	0·54	0·42	0·44	0·37
18	0·41	0·37	0·28	0·33
19	0·52	0·39	0·28	0·39
20	0·62	0·28	0·38	0·42
Mean	0·443	0·309	0·340	0·316

In each case the figures are the radial advances occurring at the four points (*a*, *b*, *c*, *d*), the highest value recorded in each case being placed first (*a*), and the rest in serial order around the circumference. The mean values show an alternation of high and low values. The mean value of the figures in column *c* is necessarily lower than the mean value of those in column *a*, since the highest values have arbitrarily been selected and placed first. From these figures it is possible to test more rigidly the relation of the resistances of opposite sides of individual apples.

In order to test this point correlations were calculated between radial advance in column *a* and the values of each of the other columns in turn. The results are as follows :—

$$\left.\begin{aligned} r_{ab} &= +0\cdot680 \\ r_{ac} &= +0\cdot742 \\ r_{ad} &= +0\cdot679 \end{aligned}\right\}.$$

All these correlations are positive and highly significant, indicating again that the resistance within an apple tends to a specific value. To test whether the alternation in high and low values in columns *b*, *c* and *d*, as indicated by the means, is significant, the effect of the variation in resistance at the point (*a*) was eliminated, so that the relations of the resistances at the points (*b*), (*c*) and (*d*) were found for apples of equal resistance at the point (*a*). The partial correlations $r_{bc.a}$ and $r_{cd.a}$ were therefore calculated with the following results :—

$$\left.\begin{aligned} r_{bc.a} &= -0\cdot102 \\ r_{cd.a} &= -0\cdot159 \end{aligned}\right\}.$$

These values are quite insignificant and indicate that in apples of equal resistance at one point no real difference on the average exists between the resistance at any two other points round the circumference. This result was confirmed by taking differences between the figures in columns *b* and *c* and *c* and *d*, and testing by Student's method. The evidence for the specific value of resistance in individual apples is thus seen to be cumulative.

The final test made was to inoculate apples at two opposite points (*a* and *c*). The invasion was allowed to reach a certain stage and then the rotten tissue was removed and weighed, and the holes sealed with wax. The apples were then reinoculated at the alternate points (*b* and *d*) and after a time the amount of rot determined as before. The results are given below in Table V.

Table V.—Radial Advance following two Successive Inoculations of same Individual Apples.

	Radial advance after first inoculation. 16 days.	Radial advance after second inoculation. 10 days.
1	0·47	0·46
2	0·46	0·31
3	0·44	0·30
4	0·40	0·30
5	0·34	0·26
6	0·34	0·29
7	0·34	0·28
8	0·27	0·22
9	0·24	0·21
10	0·24	0·22

A correlation coefficient was determined between the radial advance resulting from the first and second inoculations, respectively. The value found was $r = +0 \cdot 816$, which agrees very well with the values previously determined for correlation in resistance between the halves of single apples. The value has a significance greater than 100 to 1 (6) and finally proves the specific value of resistance in the individual apple.

There still remains the remote possibility that variations in invasion were due to changes in the fungus. To test this, the organism was re-isolated from apples showing extreme variations in attack (100 per cent. and 0·4 per cent. of rot). No marked differences could be detected in the vigour of the fungus as measured by its growth rate on a standard synthetic medium; and such differences as were found bore *no relation to the amount of rot at the point from which the fungus was re-isolated.*

The results obtained from further applications of the statistical method described in this communication will be presented in the second paper of this series, when certain specific problems connected with the resistance of apples to fungal invasion and the virulence of fungal strains will be considered.

VII.—*Summary.*

1. A standard technique is described for studying the invasion of apples of different varieties inoculated with various species of fungi. The invasion has been studied during various periods of storage life and at different temperatures.

2. The primary data are derived from upwards of 15,000 inoculations and consist of the weights of rotted tissue derived from individual apples. It is shown that, since invasion occurs in a sphere of limited size, a complex relation

holds between the weight of tissue rotted and the penetration of the fungus. In consequence of this the weight of rotted material obtained during two consecutive equal periods of time will not be equal, even though the penetration of the fungus is at a uniform rate. A method has been devised which takes into account this complex relationship, and a new measure "radial advance" is calculated which is constant over equal intervals of time when invasion is proceeding at a uniform rate, irrespective of the stage of invasion reached.

3. The percentage weights of rotted tissue derived from individuals in a sample of apparently uniform apples are found to vary over a wide range. The distribution of percentage weights with a given fungus is shown to be highly asymmetric, while the same data converted into radial advance give a distribution only moderately asymmetric. The standard error of the mean of radial advance is shown to be less than half that of percentage weights, indicating a great gain in accuracy in the use of radial advance as a measure of invasion.

4. The rate of radial advance provides a convenient scale in which to measure the resistance to invasion in individual apples.

5. It is found that each individual apple in a sample has a characteristic resistance. This is shown by :—

(1) The high positive correlation obtaining between the radial advance at the opposite sides of individual apples inoculated at two or four points :—

$$r = +0\cdot694 \text{ (2 points, 116 apples).}$$

$$r = +0\cdot742 \text{ (4 points, 20 apples).}$$

(2) By the high correlation found to hold between the radial advances obtained from two successive inoculations of the same individuals by the same fungus :—

$$r = +0\cdot816 \text{ (10 apples).}$$

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A Quantitative Study of the Course of Fungal Invasion of the Apple Fruit, and its Bearing on the Nature of Disease Resistance.—Part II. *The Application of the Statistical Method to certain Specific Problems.*

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(Communicated by Prof. V. H. Blackman, F.R.S.—Received March 31, 1927.)

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CONTENTS.

Page
I. Introduction 444
II. The time sequence of invasion for selected fungal species........ 445
III. Comparison of the virulence of certain *Fusarium* strains........ 451
IV. Comparison of variation in resistance shown by different populations of Bramley's Seedling apples........ 459
V. Concerning resistance to fungal invasion in apples........ 461
VI. Summary........ 465

I.—*Introduction.*

In the previous paper (1) a method was developed for studying quantitatively the process of invasion of apples by fungi inoculated into them. It was there shown that the method is capable of yielding a measure of the amount of invasion (radial advance) which is independent of the size of the apple used and of the time of duration of the experiment. The distribution of radial advance in populations of apples was studied and it was shown (1) that the distribution of radial advance* (in terms of which resistance to invasion is measured) is not highly asymmetrical if measured at the right stage of invasion, (2) that an individual apple is more uniform with respect to resistance than are any two apples selected at random. It is shown in the course of the present paper that by utilising radial advance as a measure of invasion the change in resistance to the attack of fungal species during the period of storage can be followed. A comparison is made of the virulence of certain fungal species or strains and of the variation in resistance to fungal invasion shown by different populations of given varieties of apples during storage. The significance of these comparisons has been estimated by the calculation of probable errors, which was shown to be legitimate in the previous paper. Finally the bearing of the results obtained on the problem of disease resistance is discussed.

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* Resistance has been assumed to be inversely proportional to the rate of radial advance.

II.—*The Time Sequence of Invasion for Selected Fungal Species.*

Some information had already been obtained in 1921–22 on the behaviour of certain selected fungi. The results attained at that time were based on periodic counts of the number of apples showing externally marked signs of invasion (using samples of 10 apples). In fig. 1 which is a selection of curves already published (2) the differences in the time of onset of the main invasion are seen to vary greatly for the different species employed, and for this reason some of these species have been selected for further work. As far as the experiment of 1921–22 went it clearly established the fact that the successional invasion of fungal species observed in apples in cold store was not due to a failure of infection, but to a check in development after infection had occurred (3). It seemed desirable, however, to investigate by a more exact method the relation between parasite and host during the period of delay in invasion.

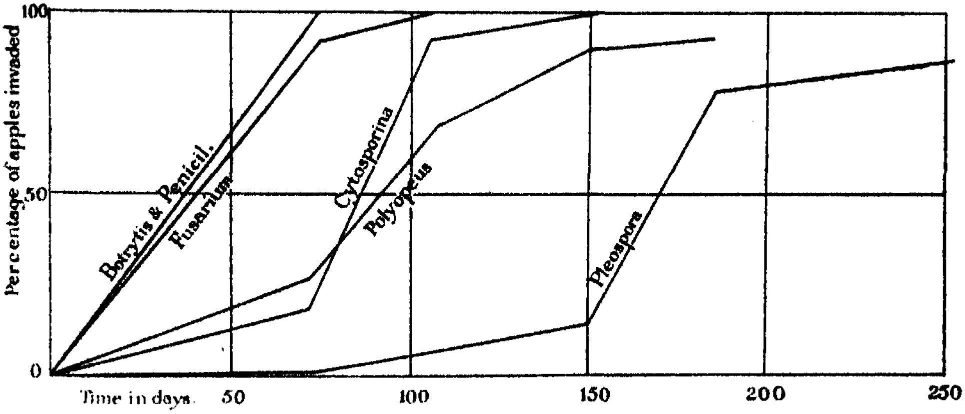

Fig. 1.—Time succession in percentage of apples in a sample showing invasion by various species of fungi. 1° C.

The species selected for further study were (1) *Pleospora pomorum*, (2) *Polyopeus aureus*, (3) *Fusarium* strain A, (4) *Cytosporina ludibunda*, (5) *Botrytis* sp. These were grown on Cox's Orange Pippin apples, obtained from Burwell, Cambridgeshire. One thousand apples were selected and divided into two batches of 500 each. Each batch was inoculated with the five specified fungi (100 apples each). With the first set the inoculations commenced on October 19 and ended on October 23, 1925, using the fungi in the order given above. This set was despatched to the Low Temperature Station, Cambridge, on

October 26 and placed at 12° C. The second set was inoculated exactly one week later, using the same fungi in the same order, and despatched to Cambridge on November 2. These a ples were stored at 3° C.

Each set of 100 apples was divided at random into 5 samples of 20 apples each before despatching the set to Cambridge. Five samples (one for each fungus) were returned to London at more or less regular intervals (fortnightly intervals for those stored at 12° C., monthly intervals for those stored at 3° C.) for systematic examination. The results of the 1925 experiment with Cox's Orange Pippin are given in the following table.

The fungal species are indicated in column 2, the Roman numerals indicating the number of the sample. The time which has elapsed since inoculation is found in column 3. Column 4 gives the mean weights of the apples at the time of sampling, and the calculated mean radius is found in column 5. In column 6 are the mean radial advances derived from the figures for percentage weight of tissue rotted in each apple of the sample, by the use of the curve in fig. 1 in Part 1 of this paper. The averages of these figures for radial advance are the mean radial advances found in column 6. Column 7 gives the percentage weight of apple rotted, derived from the primary experimental data. The product of the mean radii and the radial advances yield the figures in column 8, and are radial advances in centimetres. Dividing the figures in this column by the number of days elapsed from inoculation the growth rates per day in centimetres, found in column 9, were obtained. The mean growth rates in the intervals between sampling are found in column 10, and were obtained by dividing the increments in radial advance in column 8 by the time elapsed between the two samplings indicated.

The different levels of growth rate exhibited by the various fungi are clearly represented by the figures in columns 9 and 10 of Table I. In the case of *Botrytis*, the growth rate at the higher temperature is approximately double that of the lower, while with *Fusarium* the difference is slightly in favour of the higher temperature. The growth rate of *Polyopeus aureus* at the two temperatures is almost identical. *Pleospora* exhibits a slightly higher growth-rate at the lower temperature and this effect is very marked in the case of *Cytosporina*. With *Fusarium* " interference " occurred at both temperatures.

Averaging the growth-rates at the two temperatures, the order of diminishing values would be *Botrytis*, *Cytosporina*, *Fusarium*, *Polyopeus aureus* and *Pleospora*. This is precisely the order noted in the succession of species already referred to in fig. 1, with the exception of *Fusarium* in which case a weaker strain was utilised for this experiment. The variation in " reaction " exhibited by

Table I.—Rate of Invasion for certain Fungi at different Temperatures.

1. Temperature. ° C.	2. Sample.	3. No. of days from inoculation.	4. Mean apple in grams.	5. Mean radius in centimetres.	6. Radial advance.	7. Percentage weight of tissue rotted.	8. Radial advance in centimetres.	9. Growth rate per day in centimetres.	10. Growth rate during interval in centimetres per day.	
3	*Botrytis* I	35	63·9	2·63	0·648	24·76	1·704	0·0487	0–I	0·0487
3	,, II	68	65·9	2·65	0·800	36·24	2·12	0·0352	I–II	0·0126
3	,, III	89	54·4	2·49	1·302	91·2	3·24	0·0364	II–III	0·0533
3	,, IV	120	62·5	2·61	1·137	79·8	2·97	0·0248	III–IV	—0·0087
12	*Botrytis* I	19	68·1	2·68	0·850	47·3	2·27	0·119	0–I	0·119
12	,, II	33	67·0	2·67	1·275	89·4	3·40	0·106	I–II	—0·0807
12	,, III	47	67·0	2·67	1·247	86·1	3·33	0·071	II–III	—0·0050
12	,, IV	61	67·0	2·67	1·400	100·0	3·74	0·061	III–IV	0·0293
3	*Cytosporina* I	36	60·69	2·59	0·563	21·0	1·458	0·040	0–I	0·040
3	,, II	70	62·3	2·60	0·816	44·86	2·121	0·030	I–II	0·0195
3	,, III	90	64·3	2·63	0·964	61·0	2·54	0·028	II–III	0·0210
3	,, IV	117	65·7	2·65	1·018	63·8	2·70	0·023	III–IV	0·0059
12	*Cytosporina* I	21	64·8	2·64	0·125	0·9	0·330	0·016	0–I	0·016
12	,, II	35	64·9	2·64	0·164	0·8	0·424	0·012	I–II	0·0067
12	,, III	48	65·5	2·65	0·186	0·9	0·493	0·010	II–III	0·0053
12	,, IV	75	53·9	2·49	0·170	2·0	0·424	0·0057	III–IV	—0·0025
12	,, V	98	57·9	2·56	0·297	11·7	0·760	0·0078	IV–V	0·0146
3	*Fusarium* I	38	68	2·71	0·322	3·1	0·873	0·023	0–I	0·023
3	,, II	71	68·1		0·376	4·6	1·19	0·015	I–II	0·0096
3	,, III	92	70·9		0·38	4·6	1·19	0·013	II–III	0
3	,, IV	118	71·8		0·38	4·6	1·19	0·010	III–IV	0

Table I—(continued.)

1. Temperature.	2. Sample.	3. No. of days from inoculation.	4. Mean apple in grams.	5. Mean radius in centimetres.	6. Radial advance.	7. Percentage weight of tissue rotted.	8. Radial advance in centimetres.	9. Growth rate per day in centimetres.	10. Growth rate during interval in centimetres per day.	
° C.										
12	*Fusarium* I	22	66·89	2·67	0·336	4·4	0·897	0·0408	0–I	0·0408
12	,, II	36	68·63	2·69	0·378	5·5	1·017	0·0282	I–II	0·0086
12	,, III	49	65·4	2·65	0·296	7·0	1·155	0·0236	II–III	0·0029
12	,, IV	88	62·7	2·46	0·520	22·1	1·279	0·0145	III–IV	0·0032
12	,, V	100	47·7	2·39	0·702	36·0	1·678	0·0168	IV–V	0·0333
3	*Polyopeus* I	38	65·8	2·70	0·235	1·4	0·635	0·0168	0–I	0·0168
3	,, II	71	64·8		0·267	2·6	0·720	0·0102	I–II	0·0026
3	,, III	92	67·0		0·282	4·1	0·762	0·0083	II–III	0·0020
3	,, IV	118	77·8		0·501	21·4	1·328	0·0089	III–IV	0·00217
12	*Polyopeus* I	22	77·7	2·76	0·143	0·6	0·394	0·0179	0–I	0·0179
12	,, II	36	70·7		0·235	1·5	0·648	0·0180	I–II	0·0181
12	,, III	48	72·6		0·252	1·4	0·693	0·0144	II–III	0·0038
12	,, IV	77	60·4		0·252	2·3	0·693	0·0090	III–IV	0
12	,, V	99	53·3		0·329	10·7	0·908	0·0092	IV–V	0·0098
3	*Pleospora* I	40	77·6	2·78	0·225	1·1	0·626	0·0156	0–I	0·0156
3	,, II	74	75·9		0·160	0·7	0·446	0·0064	I–II	—0·0053
3	,, III	94	71·9		0·150	0·7	0·417	0·0043	II–III	—0·0015
3	,, IV	120	64·6		0·196	1·1	0·547	0·0046	III–IV	0·0050
12	*Pleospora* I	24	67·2	2·69	0·104	0·4	0·280	0·0117	0–I	0·0117
12	,, II	38	69·3		0·218	1·2	0·586	0·0154	I–II	0·0218
12	,, III	51	63·7		0·185	1·5	0·342	0·0067	II–III	—0·0187
12	,, IV	79	61·9		0·191	2·9	0·514	0·0065	III–IV	0·0061
12	,, V	101	58·3		0·262	3·5	0·705	0·0070	IV–V	0·0086

the ***Fusarium*** strains is dealt with later. In every case the rate of growth falls in the early part of the experiment, but in the case of ***Fusarium*** at 12° C. and of *Pleospora* at 12° C. there is an indication of a subsequent rise.

In spite of the part played by sampling errors there is an unmistakable similarity in the drift of growth-rate. The growth-rate falls rapidly in each case until the neighbourhood of the fiftieth day where a minimum occurs, and subsequently in most cases there is an increase in growth-rate. Averaging the values* in all the experiments the following figures are obtained.

Table II.—Mean Values for Growth-rate in time for the Fungi in Table I.

Days from inoculation.	Mean growth-rate in centimetres per day.
20	0·031
30	0·022
40	0·0086
50	0·0072
60	0·0074
80	0·013

The growth-rates have been assumed to be inversely proportional to the resistance of the apple to invasion, and since the growth-rates of all the fungal species employed exhibit the same kind of time-relation, irrespective of temperature, there is possibly a general change in resistance occurring in every case. It is probable that the resistance of the apples was increasing up to the fiftieth day from inoculation, with slight indication of a subsequent fall. If it is assumed that the variation in resistance with time is represented by a curve of the kind shown in fig. 2 and that L 1 and L 2 represent two critical levels of resistance for the two species of fungus F 1 and F 2, then for species F 1 the growth-rate will fall during the interval of time from 0 to T 1 (since during this time the resistance is rising) will reach a minimum somewhere between T 1 and T 2, and will subsequently rise as the resistance once again falls. Such a distribution of growth-rates is seen in the case of *Botrytis* at 3° C. In the case of species F 2 the growth-rate will fall rapidly up to a time T 1 at which point the resistance rises above the critical level. Growth will then cease until such time as the resistance again falls below the critical value. If the portion of the apple which has been already invaded before the first critical point is reached is not negligibly small when growth is checked, subsequent drying out may lead to development of cavities which are characteristic of the phenomenon of "interference." This effect was very evident with *Fusarium* at both temperatures and sporadically with *Polyopcus aureus*. In the case of slow-growing fungi the volume of the apple affected before the growth ceases would be so small that the cavities would not be evident.

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* The values on the particular days were obtained by interpolation from curves.

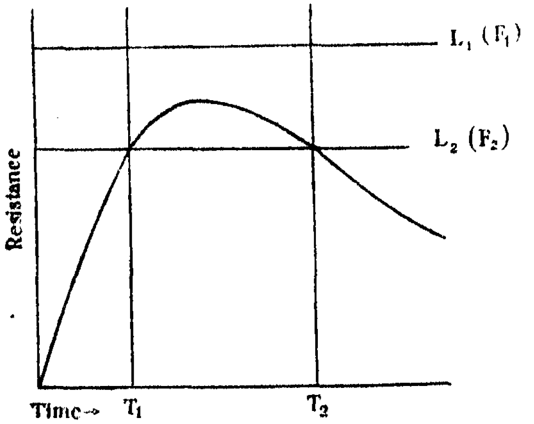

Fig. 2.

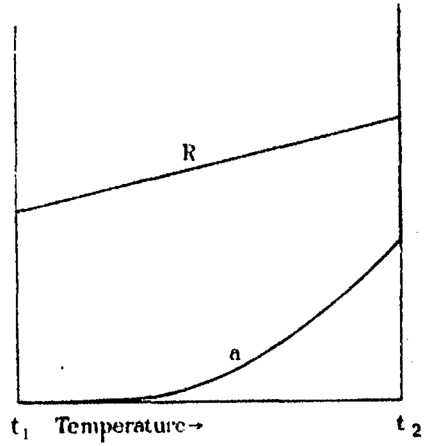

Fig. 3.

The varying reactions of the fungi at the two temperatures is striking and is difficult to interpret. The relation between parasite and host will depend on the effect of temperature, on the activity of the fungus, and the resistance of the apple. For *Botrytis*, which is a virulent species, the activity is found to increase to double for the temperature rise of 9°, which is near the normal temperature coefficient of growth. In the case of *Fusarium* and the other fungi, the increase in activity is not evident. It is possible that it is masked by the relatively higher resistance of the apple in these cases.

Should the temperature coefficients of resistance and activity be greatly different, the temperature relations may be formally represented by the diagram shown in fig. 3. The rate of invasion has been assumed to be proportional to $1/R$, where R is the resistance of the apple. If a is the activity of the fungus we may write

$$V = \frac{a}{R + C}$$

where V is the rate of radial advance and C a constant. It is clear that if the relations indicated in the diagrams hold, $a/R + C$ may have a minimum value between temperatures T 1 and T 2. Such a set of relationships might account for the results obtained with *Cytosporina* at 12° C. and 3° C.; and by suitable modification of the temperature effect on R, such cases as *Fusarium* and *Polyopeus aureus* would be explicable. With our present knowledge it is impossible either to substantiate or refute such a hypothesis, but further lines of work are clearly indicated. The evidence to hand as to the nature of resistance will be discussed later in this paper.

III.—*Comparison of the Virulence of Certain Fusarium Strains.*

An interesting aspect of the problem of invasion of apples is the relative degree of virulence of various strains of a single species of fungus. In the preceding section of this paper different species have been compared, and similar methods will now be applied to various strains of a given species. As some of the strains studied are saltant forms whose origin and previous history are well known, it has been possible to compare these with their parent strains. The practical importance of such comparison in relation to the possible origin of new diseases of apples is evident.

The *Fusarium* strains used for these experiments were originally isolated from diseased apples. The strain A was obtained by Dr. W. Brown of this College, in 1918, from an unknown variety ; the strains B, C, and D from Cox's Orange Pippin, E from Allington Pippin, and F from Sweet Alford, in 1920 by one of the present authors (A.S.H.). The strains B 1, C 1 and C 2 originated from monosporous platings of B and C respectively, and were made in 1921. C 3 was first obtained as a variant from C in tube cultures made from the same monosporous colony of C. The strains B 11, B 111 and D 2 are saltants obtained by Brown, who has recently dealt with the question of saltation in some detail (4). The origin of the strains and saltants is shown diagrammatically below (fig. 4).

The fact that this species of *Fusarium* was capable of parasitising the tissues of the apple was first shown in 1921 (3, p. 369) when, with the exception of *Penicillium* and *Botrytis*, it proved at 1° C. the most active fungus tested in that year (see fig. 1). In 1922 eight strains were tested on the Cox's Orange Pippin and Bramley's Seedling varieties of apples at two temperatures (1° C. and 15° C.). The strains included A, C, C 1, C 2, B 1, D, E, and F. All the strains employed invaded both the varieties at 15° C., but the intensity of attack was much less marked in the case of the Bramley's. At 1° C., the Cox's were slightly invaded by the strains C, C 1, C 2, F, D, B 1, and E. The invasion of the Bramley's at this temperature was negligible (5, p. 39, and 6, p. 59).

As a result of experiments begun in 1923 using the strains C 1, C 2 1, C 3, B 1, B 1 1, A and D on Cox's Orange Pippin at two different temperatures (12° C. and 3° C.) not only was the parasitic activity of these *Fusarium* strains con-

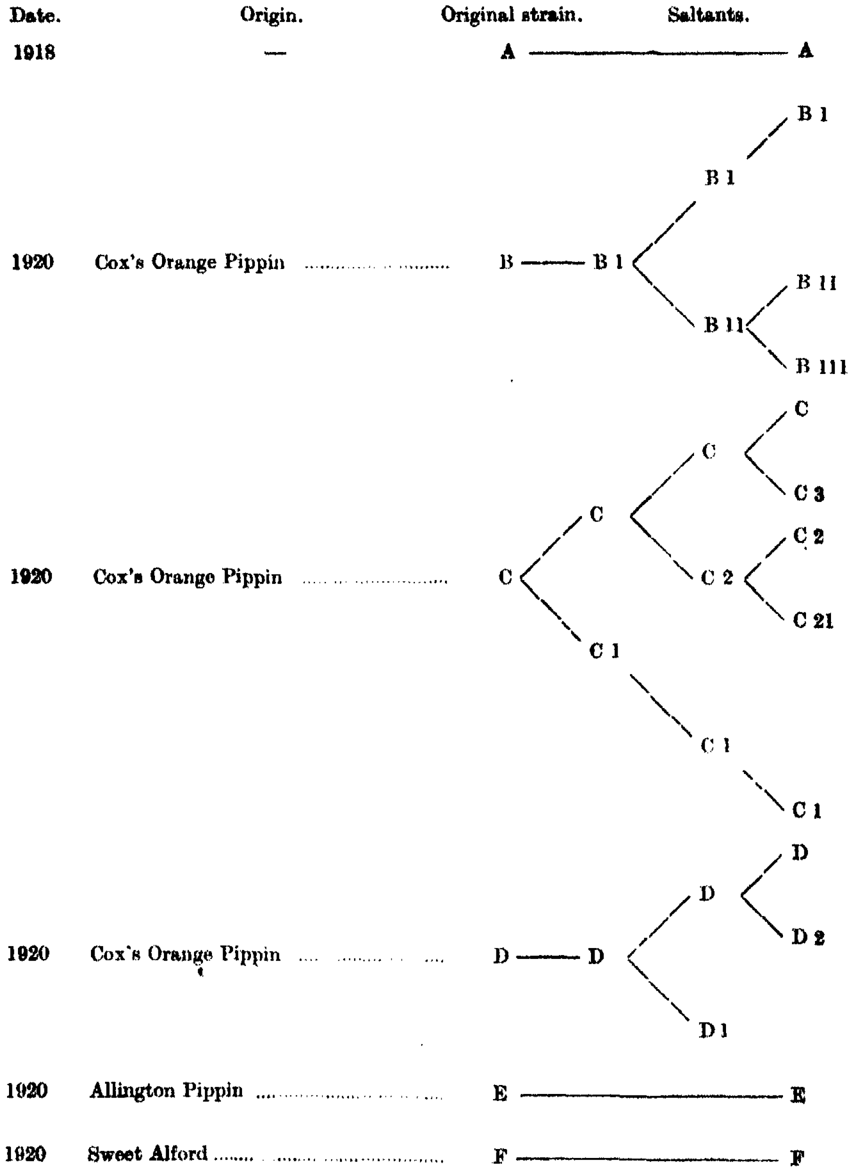

FIG. 4.—Pedigree of *Fusarium* strains.

firmed, but in addition it was clearly evident that they varied in their pathogenicity (6, p. 62). Certain strains such as D 1, C 1, C 21, and B 1 being very active, others (A, C 3) moderately active and others (B 11) very weak. It was found that the strong strains belonged to the group of strains which exhibit strong mycelial development, whereas the weak strains were those characterised by feeble mycelial development and intense sporulation (see (7)). It was also clear that the saltant B 11 derived from the strongly active strain B 1 differed from the parent in a marked loss of virulence.

In 1924 and 1925 the same experiments were repeated on a larger scale, with Bramley's in addition to the Cox's, using the more exact methods for recording data already outlined in this paper. The results obtained in both years confirm the earlier work, and evidence will be presented to show that the strains can be arranged in serial order with respect to their pathogenicity, and that the sequence is independent of the type of apple used.

The data obtained are summarised in Table III in which the rate of advance in centimetres per day is given in column 6 for each of the strains of *Fusarium* given in column 1. The probable errors for the rates of advance have been calculated and the significance of the differences between strains can thus be tested.

Table III.—Rates of Invasion for certain *Fusarium* Strains in two Varieties of Apple.

1. *Fusarium* strain.	2. Mean weight of apple in grams.	3. Mean radius in centimetres.	4. Radial advance.	5. Radial advance in centimetres.	6. Rate of advance per day in centimetres.
Bramley's Seedling, 1925–26. 12° C. 70 days.					
A	156·2	3·66	0·557 ±0·030	2·04 ±0·15	0·0292 ±0·0021
B 11	153·9	3·65	0·337 ±0·017	1·23 ±0·065	0·0178 ±0·0009
B 111	137·8	3·51	0·356 ±0·021	1·25 ±0·077	0·0179 ±0·0010
C 1	141·0	3·53	0·469 ±0·042	1·66 ±0·15	0·0237 ±0·0021
C 21	145·8	3·58	0·486 ±0·043	1·74 ±0·17	0·0248 ±0·0024
C 3	147·8	3·60	0·523 ±0·046	1·88 ±0·17	0·0296 ±0·0024
D	174·9	3·81	0·595 ±0·036	2·26 ±0·14	0·0322 ±0·0020
D 2	145·3	3·58	0·367 ±0·018	1·31 ±0·068	0·0188 ±0·0010
		Mean P.E. ±0·056			
Bramley's Seedling, 1924–25. 12° C. 103 days.					
A	91·3	3·07	0·378 ±0·041	1·160 ±0·13	0·0113 ±0·0013
B 1	90·9	3·06	0·606 ±0·038	1·854 ±0·13	0·0180 ±0·0013
B 11	115·5	3·05	0·414 ±0·043	1·263 ±0·13	0·0123 ±0·0013
B 111	99·5	3·15	0·131 ±0·027	0·413 ±0·11	0·0040 ±0·0011
C 1	101·2	3·17	0·754 ±0·054	2·39 ±0·17	0·0232 ±0·0017
C 21	106·8	3·23	0·622 ±0·037	2·009 ±0·12	0·0195 ±0·0012
C 3	88·1	3·03	0·652 ±0·048	1·976 ±0·15	0·0192 ±0·0015
D	100·2	3·16	0·714 ±0·046	2·256 ±p·15	0·0219 ±0·0015
		Mean P.E. ±0·048			

Table III—(continued).

1. *Fusarium* strain.	2. Mean weight of apple in grams.	3. Mean radius in centimetres.	4. Radial advance.	5. Radial advance in centimetres.	6. Rate of advance per day in centimetres.
Cox's Orange Pippin, 1924–25. 12° C. 54 days.					
A	66·54	2·66	0·860 ±0·062	2·288 ±0·166	0·0424 ±0·0031
B 1	66·13	2·66	0·952 ±0·080	2·532 ±0·214	0·0469 ±0·0040
B 11	67·59	2·68	0·380 ±0·037	1·018 ±0·099	0·0188 ±0·0018
B 111	64·77	2·64	0·103 ±0·023	0·272 ±0·061	0·0050 ±0·0011
C 1	59·33	2·57	1·025 ±0·063	2·634 ±0·264	0·0487 ±0·0030
C 21	70·06	2·71	0·953 ±0·024	2·583 ±0·065	0·0478 ±0·0012
C 3	68·11	2·69	0·786 ±0·068	2·114 ±0·184	0·0392 ±0·0034
D	64·01	2·63	1·038 ±0·049	2·730 ±0·132	0·0506 ±0·0025
		Mean P.E. ±0·027			
Cox's Orange Pippin, 1924–25. 3° C. (corrected for interference). 138 days.					
A	68·7	2·74	0·531 ±0·046	1·455 ±0·128	0·0105 ±0·00093
B 1	61·7	2·64	0·747 ±0·069	1·972 ±0·184	0·0143 ±0·0010
B 11	66·7	2·71	0·194 ±0·043	0·526 ±0·117	0·00381 ±0·00085
B 111	72·4	2·78	0·016 ±0·011	0·046 ±0·031	0·00033 ±0·00023
C 1	68·6	2·74	0·618 ±0·061	1·693 ±0·169	0·0122 ±0·0012
C 21	68·0	2·73	0·587 ±0·062	1·603 ±0·171	0·0116 ±0·0013
C 3	62·2	2·65	0·813 ±0·054	2·154 ±0·142	0·0156 ±0·0010
D	73·1	2·79	0·598 ±0·077	1·668 ±0·216	0·0121 ±0·0016
		Mean P.E. ±0·036			
Cox's Orange Pippin, 1925–26. 12° C. 89 days.					
A	62·7	2·46	0·541 ±0·044	1·33 ±0·109	0·015 ±0·0012
B 11	60·6	2·44	0·240 ±0·020	0·59 ±0·049	0·0066 ±0·00055
B 111	67·8	2·53	0·239 ±0·0088	0·60 ±0·024	0·0067 ±0·00027
C 1	64·4	2·44	0·800 ±0·023	1·95 ±0·101	0·022 ±0·0011
C 21	54·5	2·35	0·750 ±0·072	1·76 ±0·187	0·020 ±0·0021
C 3	57·4	2·40	0·520 ±0·075	1·25 ±0·181	0·014 ±0·0020
D	60·9	2·44	0·840 ±0·023	2·05 ±0·062	0·023 ±0·00069
D 2	63·1	2·47	0·353 ±0·052	0·87 ±0·129	0·0098 ±0·0014
		Mean P.E. ±0·033			

The data of rate of advance have been presented again in serial order in Table IV, the most rapidly growing strain being in each case in the first column.

From Table IV it is clear that the two experiments with Cox's Orange Pippin agree exactly in the order of virulence of the strains, when D 2 and B 1 which were not common to the two experiments are left out of account. This agreement is the more striking when it is observed that the growth-rate in the 1925–26 set is very much less than for 1924–25.

Table IV.—Rates of Invasion for the same *Fusarium* Strains arranged in serial order.

	Strains of *Fusarium* and radial advance in centimetres per day.							
Bramley's Seedling, 1925–26. 12° C. 70 days.	D 0·0322 ±0·0020	C 3 0·0296 ±0·0024	A 0·0292 ±0·0021	C 21 0·0248 ±0·0024	C 1 0·0237 ±0·0021	D 2 0·0188 ±0·0010	B 111 0·0179 ±0·0070	B 11 0·0178 ±0·0009
Bramley's Seedling, 1924–25. 12° C. 103 days.	C 1 0·0232 ±0·0017	D 0·0219 ±0·0015	C 21 0·0195 ±0·0012	C 3 0·0192 ±0·0015	B 1 0·0180 ±0·0013	B 11 0·0123 ±0·0013	A 0·0113 ±0·0013	B 111 0·0040 ±0·0011
Cox's Orange Pippin, 1925–26. 12° C. 89 days.	D 0·023 ±0·00069	C 1 0·022 ±0·0011	C 21 0·020 ±0·0021	A 0·015 ±0·0012	C 3 0·014 ±0·0020	D 21 0·0098 ±0·0014	B 111 0·0067 ±0·00027	B 11 0·0066 ±0·00055
Cox's Orange Pippin, 1924–25. 12° C. 54 days.	D 0·0506 ±0·0025	C 1 0·0487 ±0·0030	C 21 0·0478 ±0·0012	B 1 0·0469 ±0·0040	A 0·0424 ±0·0031	C 3 0·0392 ±0·0034	B 11 0·0188 ±0·0018	B 111 0·0050 ±0·0011
Cox's Orange Pippin, 1924–25. 3° C. 138 days.	C 3 0·0156 ±0·0010	B 1 0·0143 ±0·0010	C 1 0·0122 ±0·0012	D 0·0121 ±0·0016	C 21 0·0116 ±0·0013	A 0·0105 ±0·00093	B 11 0·0038 ±0·00085	B 111 0·0033 ±0·00023

The agreement between the corresponding Bramley's series in relation to order of virulence is much less marked. For instance C 1 in 1924–25 occupied the first place while in 1925–26 it was fifth in order. Strain A also has changed from being the last but one in 1924–25 to the third place in 1925–26. Similar discrepancies are seen in the set of *Fusarium* strains growing in Cox's Orange Pippin at 3° C. as compared with those at 12° C.

The records made at the time of estimating the rot show that signs of withering were noted in both the sets with Bramley's Seedling and the Cox's at 3° C. In the case of the Bramley's 1924–25 set and the Cox's 3° C., notes with regard to withering were made on each individual apple, and it was possible in these cases to arrive at an estimate by noting the proportion of apples which showed signs of withering. As it seemed possible that the withering had partly accounted for the changed order in these cases, as compared with the Cox's at 12° C., correlations were worked out in each case between the percentage of withered apples in a set and the growth-rates. The results gave for Bramley's 1924–25, $r = -0\cdot833$ and for Cox's $r = -0\cdot666$. The negative sign in both cases indicates that the growth-rate tends to fall with increasing intensity of withering.

As it was thought that these correlations may have been due to a spurious effect—since in general the greatest withering occurred with the slowest growing strains, and the possibility of the absorption of water by the living tissue from the dead watery areas could not be neglected—another method of correlation was resorted to. In this case the rates of advance calculated for the set of Bramley's 1925–26 where withering was not so marked, and the set of Cox's 1924–25, 12° C., where withering was not noted, were taken as normal. The ratios of the growth-rates of these sets as compared with corresponding abnormal sets were determined and their ratios correlated with the observed percentage of withering. The correlation coefficients were for Cox's $r = +0\cdot738$ and for the Bramley's $+0\cdot789$. The positive sign of this result indicates that where the percentage of withering was high, the ratio of the rate of growth in the normal set as compared with the abnormal set tended to rise; or, in other words, in the withered apples the growth-rate of the fungus was suppressed.

As in this method the rates in apples affected with the same strain were compared, there is no question of a differential effect on water loss due to the presence of different amounts of rotten tissue. The evidence seems to point to a real effect of withering on the growth-rate of the fungus, and may possibly arise by competition for water between the fungus and the host tissues, or an increase in concentration of the cell contents. The drying-out of the rotten areas, in cases where "interference" occurs, indicates that absorption of water from the dead tissues can actually occur, and experiments to test the effect of water loss are now in progress.

In order to assess the relative effects of apple variety, of season, and of temperature on the rate of invasion by different strains of *Fusarium*, Fisher's method of analysis of variance (8) was applied to the data. It was assumed that the rate of radial advance is determined by the interaction of two factors, viz., the susceptibility of the apple and the activity of the strains, and also that the rate of advance was proportional to the products of these two factors. For this reason in the process of analysis the logarithms of the values for radial advance were used instead of the actual values recorded. Seven strains were investigated, D 2 and B 1 being omitted since the records for these two strains were not complete. Variety of apple and experimental conditions (season and temperature) yielded five combinations. Thus there were in all 35 combinations of experimental conditions with fungal strains giving 34 degrees of freedom, 7 fungal strains with 6 degrees of freedom, and 5 experimental conditions with 4 degrees of freedom, respectively. The results of the analysis are shown in Table V.

Table V.—Analysis of Variants.

	Degrees of freedom.	Variance.	Mean square.
Strains	6	2·68179	0·44699
E. conditions	4	1·40910	0·35228
Remainder	24	1·61746	0·06740
Total	34	5·70835	—

The 24 remainders give an estimate of the mean discrepancies of individual results from the assumed product formula. The figure corresponds to a standard error of 16·7 per cent. for a single determination. From the figures for probable error of radial advance in Table III it is possible to compare this calculated mean error with the experimental errors actually recorded, and thus to test whether the departures from the product formula were significant. For strain D the mean error recorded in the experiments was 11·4 per cent., for strain C 3 14·0 per cent., while for strain B 111, in which variation in radial advance was greatest, the value of the mean error was 29·7 per cent.

It is clear then that the errors recorded in the experimental data agree very well with the expected discrepancy from values for radial advance based on the assumption that this process is conditioned by the product of activity of fungal strains and susceptibility of apples—susceptibility is taken to be the reciprocal of resistance.

Table V shows further that the differences in strains had the largest effect on rate of advance, but that the experimental conditions were almost as effective. There is no significant difference between these two effects, but both show very high significance (about eight times their mean error) as compared with the random errors.

The effect of experimental conditions can be traced almost entirely to the difference between the experiments at 12° C. and the single experiment at 3° C. The seasons and variety of apple show no significant differences in their effects among themselves. It may be concluded from this that the variety of apple and seasonal effects do not greatly affect the relative virulence of the different strains tested, while there are indications that at different temperatures the amount of infection due to different strains relative to each other may vary. The effect of different levels of temperature on the relative virulence of fungal species has already been observed, and it appears that similar relations may hold for strains of the same species.

This analysis of variance indicates further that in general the effect of the various fungal strains is due largely to the two strains B 11, B 111, which differ significantly from the rest, while in the data no significant difference among the others is found. Taking the rate of radial advance of the highest strain as 100 and calculating the others as a percentage for each set, the averages for each strain based on all the data are given in the following table (Table VI).

Table VI.—Comparison of Rates of Radial Advance for *Fusarium* Strains.

Strain.	Relative figure of radial advance.	Strain.	Difference in radial advance.
D	94·4 ±3·3		
C 1	88·7 ±3·3	D—C 1	5·7 ±4·7
B 1	87·5 ±4·6	D—B 1	6·9 ±5·7
C 21	83·4 ±3·9	D—C 21	11·0 ±5·1
C 3	82·6 ±3·7	D—C 3	11·8 ±5·0
A	72·1 ±3·1	D—A	22·3 ±4·5
D 2	50·5 ±4·3	D—D 2	43·9 ±5·4
B 11	39·7 ±2·6	D—B 11	54·7 ±4·2
B 111	22·7 ±1·5	D—B 111	71·7 ±3·6

The probable errors for each value are also given, together with the probable error of the difference between the rates of advance of the different strains as compared with strain D.

If three times the probable error is taken as significant it is seen that strains D, C 1, B 1, C 21, and C 3 do not differ significantly, while the rest, viz., A, D 2, B 11 and B 111, fall into a separate class. Furthermore, B 11 and B 111 differ significantly from strains A and D 2 and also *inter se*. The strains may then be divided into three groups D to C 3 of high, and A and D 2, medium, and B 1 and B 111 low virulence.

The history of the B strains is very interesting, for with each saltation the virulence is seen to diminish. The same relation holds for D and D 2 and also C 1, C 21 and C 3. In the last case cited the difference in virulence is not high enough to be significant, but the fact that this order is identical in the two experiments with Cox's at 12° C., as well as for the average of all, indicates strongly that, in fact, the virulence of these three strains falls into the order given. In the case of the D strains, the saltant is found in the medium class, while for the B strains in spite of the fact that B 1 is in the highest class, both saltants fall into the lowest class.

A similar relation has been found to hold with certain strains of the section *Discolor* of *Fusarium*, recently investigated in this laboratory by Horne and Mitter (9). It is perhaps significant that, in all cases of saltation whose history is known, the parent form displays a higher virulence than the strains derived from it, although the fall in virulence may vary greatly in magnitude.

IV.—*Comparison of Variation in Resistance shown by different Populations of Bramley's Seedling Apples.*

Experiments were started in the season 1922–23 to investigate the resistance of apples derived from different localities in relation to a particular fungal species. The variety of apple chosen was Bramley's Seedling and the fungus was *Cytosporina ludibunda*. The apples were obtained from two localities, viz., Burwell, Cambridgeshire (fen soil) and Spalding (silt soil). Batches of 100 apples each were inoculated and stored at Cambridge under constant conditions of humidity and temperature (1° C.). After some months the apples were examined and separated into the classes invaded and not invaded. Since "internal breakdown" (a functional disease encountered when apples are stored under low-temperature conditions) was present, the number showing signs of this trouble was also recorded. The result is given in the following table (Table VII).

Table VII.—Effect of Locality of Origin of Apples on Invasion, 1922–1923.

Locality.	Percentage of apples invaded by *Cytosporina*.	Percentage of apples invaded by *Cytosporina* and affected with internal breakdown.	Percentage of apples with internal breakdown.	Percentage of apples not invaded.	Interval in days.
Burwell (fen)	0	15	77	8	177
Spalding (silt)	32	18	10	40	237

It will be seen that the two categories of Bramley's yielded different results. Thus only 15 of the Burwell apples were invaded, whereas 50 of the Spalding apples showed signs of invasion. These figures are misleading, since of the fen apples which were affected a large percentage gave large amounts of rot, while silt apples showed very small amounts, and indeed all showed "interference." The need for an improvement on the method of assessing the resistance by the mere counting of numbers invaded became obvious at once and led to the development of the technique described in the previous paper (1).

In the following year, 1923–24, apples from six localities were obtained and stored after inoculation at 3° C. In addition to noting those apples which showed no apparent invasion and those completely rotten, the number in the intermediate class was recorded and measurements were made of the rotten areas. The results as obtained in 1923–24 are recorded in Table VIII.

Table VIII.—Effect of Locality of Origin of Apples on Invasion, 1923–1924.

Locality.	Percentage completely rotten.	Percentage intermediate stages.	Percentage not invaded.	Percentage with internal breakdown.	Interval in days.
Spalding (silt)	3	18	72	98	164
Wisbech (fen)	3	59	38	—	140
Worcester (old red sandstone)	10	84	6	—	120
Canterbury (gravel)	36	56	8	—	118
Burwell (chalk)	45	55	0	—	122
Burwell (fen)	62	38	0	—	116

Before the new measure (radial advance) could be utilised for this set of data, it was necessary to be able to convert the measurements of area into radial advance. This was done by means of two sets of data collected in the years 1924–25 and 1925–26, relating to Bramley's Seedling from Canterbury and from Burwell, for which both measurements of area and the weights of rotten tissue were recorded; 137 measurements of area and weight records were obtained. The percentage weights of rotten tissue were converted into radial advances from the graph (fig. 1) given in the previous paper (1), and a correlation diagram was set up showing the relation between radial advance (measured as percentage of radius) and the mean diametral arcs of the areas. The regression line was found to be linear, and to be practically identical for the figures derived from the two sets of apples. The correlation coefficient estimated from all the figures was $r = +0\cdot924$. From the regression line the percentage radial advance corresponding to any given area could be read off. In this way the data for areas measured in 1923–24 were converted into radial advances, and the results obtained for the apples from the six localities are given in Table IX.

The figures show large differences in the rate of radial advance for apples from different localities, and this confirms the empirical results already presented in Table VIII. Chemical analysis of samples of apples from the same localities gathered at the same time are available and have been published by H. K. Archbold (10) and Janet W. Brown (11). It is therefore possible to investigate the question as to whether chemical composition plays any part in deter-

Table IX.—Data of Invasion in Table VII in terms of Radial Advance.

Locality and soil.	Percentage radial advance.	Average percentage weight rotten.	Days.	Radial advance centimetres per day.
Spalding (silt)	0·0498	$<$ 1·0	164	0·0010
Wisbech (fen)	0·336	3·5	140	0·0084
Worcester (old red sandstone)	0·722	27	120	0·0201
Canterbury (gravel)	0·832	39	118	0·0247
Burwell (chalk)	0·977	60	122	0·0277
Burwell (fen)	1·104	87	116	0·0333

mining the relative rate of advance in the sets of apples. The rate of advance was first correlated with the nitrogen and potassium content, using the fresh weight figures in the papers already referred to. The result is as follows :—

$r_{RN} = +0{\cdot}7622$ R = radial advance per day.
$r_{RK} = -0{\cdot}3951$ K = percentage K in fresh weight of apples.
$r_{NK} = -0{\cdot}2896$ N = percentage N in fresh weight of apples.

There was found to be no correlation with the phosphate content of the apples.

The negative correlation between nitrogen and potassium in the series of apples used indicated the necessity for eliminating the effects of the association of these two factors in determining the correlation of each with radial advance. The partial correlations are shown below :—

$$r_{RN.K} = +0{\cdot}737 \qquad r_{RK.N} = -0{\cdot}282.$$

These correlations show that when the effect of the negative correlation of radial advance with potassium content has been allowed for, a large positive correlation of radial advance with nitrogen content is found, while the correlation with potassium is slightly negative, when the effect of nitrogen is eliminated. The high positive correlation of the rate of advance with nitrogen is suggestive, but not significant, since only six pairs of values were available. The negative correlation with potassium is not significant. The results indicate that high nitrogen content of apples enhances the susceptibility to the fungus used, while high potash content tends to increase resistance. The need for further investigation along these lines is evident and further work is now in progress.

V.—*Concerning Resistance to Fungal Invasion in Apples.*

Reviewing the results presented in this and the former communication it is seen that (1) the resistance of the individual apple is of a specific nature ;

(2) apples of a given variety differ in their resistance to different species of fungus and strain of a given fungus ; (3) variation in resistance of a given type of apple is associated with the season in which the apples are grown.

From a consideration of these facts, it seems reasonable to suppose that resistance is of a complex nature. It has been shown that resistance may be correlated with chemical composition of apples, and evidence has been presented which indicates that water content also plays a part.

The differences in resistance of varieties can to some extent be correlated with their known differences, such as variation in acidity, the percentage and nature of the sugars, variation in pectic content, and so on. Preliminary experiments to ascertain the effect of acidity have been carried out by growing various species of fungi in extracts of known *p*H value from apples of different varieties. The results, although merely qualitative, do show differences between the behaviour of different fungi when grown in extracts of known acidity. Two experiments were made, one with apple extracts which were made at the time of picking, and a second approximately six months later, with apples from the same original batch which had been kept at 1° C. The result of the first experiment has been published elsewhere (2, p. 50, Table I).

From this experiment it appeared that *Penicillium* and *Botrytis* grew in all the extracts, which agrees well with their known behaviour on apple varieties, while *Cytosporina*, *Fusarium* and *Pleospora* did not grow in any of the Bramley's Seedling extracts, although their behaviour differed among themselves in the extracts from the other varieties. The case of *Cytosporina* is interesting, in that growth was found in some of the Bramley's extracts made after six months' storage, when the *p*H was found to have fallen, which indicates the possibility of changes in resistance due to some tissue change in the apple. The experiments also indicated that there is a limiting level of acidity for particular species beyond which growth cannot proceed.

The relation of the growth of *Pleospora pomorum* in apple extracts of increasing acidity is brought out well by the experimental data obtained in 1921 with extracts of apples kept at 20° C. (3, p. 371). The results show increasing inhibition of growth with increasing acidity.

Further evidence indicating the same effect was obtained by inoculating apple varieties of known *p*H value and noting which varieties were infected with *Pleospora*. Sweet Alford, *p*H 4·1 — 4·6, Dymock Red, *p*H 3·9 — 4·0, Allington Pippin, *p*H 3·05 — 3·35, were invaded, whilst Bramley's Seedling, *p*H 3 — 3·1, was unaffected (see 3, p. 371).

The behaviour of *Pleospora* growing in extracts—(see 7, p. 50, Table I)—

differs from the behaviour of the fungus when inoculated into apples, in that under the latter circumstances no growth is found with Cox's Orange Pippin until the expiry of a considerable period of time (see fig. 8). This may perhaps be related to the ascertained fact that *Pleospora* is practically incapable of inverting cane sugar. The following table (Table X) shows the differences of growth of *Fusarium*, *Pleospora*, and *Cytosporina*, in liquid nutrient media containing various sugars as the source of carbon. Figures for the amount of sugar destroyed and amount of cane sugar inverted are also given.

Table X.—Effect of Sugars on Growth of certain Fungi in Culture Solution.

Fungal species.	Solution.	Weight of mycelium.	Sugar inverted.	Sugar destroyed.
		gms.	mgms.	mgms.
Fusarium	Sucrose + nutrient	0·1187 0·1532	340	45
	Glucose + nutrient	0·1041 0·1177	—	358
	Fructose + nutrient	0·0401 0·0393	—	156
Pleospora pomorum	Sucrose + nutrient	0·0085 0·0108	16·8	9·5
	Glucose + nutrient	0·0552 0·0436	—	125
	Fructose + nutrient	0·0190	—	99
Cytosporina ludibunda	Sucrose + nutrient	0·2148	271·0	33·6
	Glucose + nutrient	0·2083	—	935

The differences between *Fusarium*, *Cytosporina* and *Pleospora* with respect to the inversion of cane sugar are very striking, as is also the different amount of growth made by the three fungi in a cane sugar medium.* From such experimental evidence, the importance of the nature of the sugars present and the changes which these sugars undergo cannot be overlooked. It seems possible that the growth of *Pleospora* in the extracts derived from Cox's Orange Pippin in the table previously published ((2), p. 50, Table I) may have been due to inversion of cane sugar during the process of sterilising the medium.

The evidence taken altogether suggests very strongly that the immediate cause of resistance in different varieties of apple may vary; the resistance, for instance, shown by Bramley's Seedling may be due largely to high acidity, while the resistance of Cox's Orange Pippin may be due to the fact that in these apples the ratio of cane to invert sugar is higher than for Bramley's, or to other chemical factors. Indeed the resistance of any one variety, or any individual apple, will be determined by the interaction of all the known factors, such as, (1) acidity, (2) the nature of the sugars, (3) the percentage of nitrogen, (4) the percentage of potash, (5) the rate of loss of water, as well as possible other factors at present unknown. In so far as the intensity of these factors changes during the period of storage the resistance of individual apples to individual fungi will also show changes.

That such changes do occur is indicated strongly by the data for growth in Cox's Orange Pippin (Table I). The precise formulation of the factor-changes is at present not possible, and awaits more closely correlated data of invasion of apples by specific fungi and concurrent chemical analysis made at various times during storage; an investigation is proceeding along these lines. With our present knowledge it may be stated, as a well ascertained fact, that the acidity falls during the period of storage, so that the time of onset of invasion for a fungus intolerant of acid may be delayed until a critical level of acidity has been passed. Such a case seems to be indicated by the behaviour of *Cytosporina*, where growth did not occur in extracts from Bramley's Seedling apples until the fruit had been stored for a period of six months. Even when invasion occurs early in the period of storage, in Bramley's Seedling, which is characterised by high acidity, the decreasing acid content and consequent lowering of resistance will be reflected in an increasing rate of radial advance.

Experiments now in progress show conclusively that for apples inoculated with *Cytosporina ludibunda* and kept at 20° C. such a difference of resistance exists between those picked early and those picked late in the season. This fall of resistance in the case of Bramley's Seedling contrasts with the indicated rise in resistance for the Cox's Orange Pippin shown in Table II. Early and late picking is found in the case of Cox's to lead to a difference of resistance against the invasion of *Fusarium* and *Pleospora*, but in this case the later picked apples are found to have *a higher resistance*, which is in accordance with the estimate of change in resistance found by the measurements of radial advance. Evidence may be cited for the increase in resistance with the date of picking from early data obtained with *Pleospora pomorum* in 1922–23 (see Table XI).

Table XI.—Effect of Date of Gathering on Invasion by *Pleospora pomorum*.

Date of gathering.	Per cent. invaded.	Days in store.
October 2	69	168
October 26	20	155

The interaction of two such factors as water content and acidity—the high values of the first favouring invasion, and of the second inhibiting invasion; both factors, however, changing with time, may lead to those postulated changes in resistance which were schematically presented in fig. 2, in the attempt to account for the phenomenon of "interference."

It may be claimed for the method adopted in this paper that considerable light has been thrown on the problem of resistance of apples to the invasion of fungi. The difficulty of the problem is apparent, but not insuperable; new lines of work have been opened up, and are being actively pursued, which promise to yield results both of immediate economic importance and of scientific interest.

VI.—*Summary.*

1. The rate of invasion in an apple of a given variety does not remain constant, but may rise or fall in time, according to the variety used and the experimental conditions.

2. A comparison of *Fusarium* strains on various apples shows that the virulence of the strains falls into an order which is independent of the variety of apple and the seasonal changes, but is affected by the temperature of storage. All the saltants examined exhibited a lower virulence than the original strains from which they were derived.

3. The influence of the locality of origin on the resistance of apples of a given variety is shown to be very marked. These differences in resistance can be correlated with differences in chemical composition of the apples.

$$\begin{array}{l} r_{\mathrm{RN.K}} = +\,0\cdot737 \\[1ex] r_{\mathrm{RK.N}} = -\,0\cdot282 \end{array} \left\{ \begin{array}{l} \mathrm{R} = \text{radial advance.} \\ \mathrm{K} = \text{percentage K in fresh weight of apples.} \\ \mathrm{N} = \text{percentage N in fresh weight of apples.} \end{array} \right.$$

4. Resistance to fungal invasion is complex in nature, and depends on many factors. Some of these are water content, acidity, nitrogen content, potassium content. Low water content, high acidity, high potash content and low nitrogen content are found to be associated with high resistance. The converse is also true.

In conclusion the authors wish to express their indebtedness to Prof. V. H. Blackman for the great interest he has taken in this investigation; to various research workers of the Food Investigation Board, both in this Institution and at Cambridge, for assistance in various ways, and to Dr. R. A. Fisher of the

Rothamsted Experimental Station, for his advice on the statistical side of the investigation.

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Experimental Researches on Vegetable Assimilation and Respiration. XVII.—*The Diurnal Rhythm of Assimilation in Leaves of Cherry Laurel at "Limiting" Concentrations of Carbon Dioxide.*

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(Communicated by Dr. F. F. Blackman, F.R.S.—Received October 29, 1927.)

Section I.

(*a*) *Introductory.*

The primary general relation between external supply of carbon dioxide and rate of carbon assimilation in light by the green cells of plants has been investigated by many plant physiologists, and in general, so long as the assimilation is appreciably below the maxima permitted by the light intensity employed and by the temperature of the green cell, the relationship may be held to approximate to direct proportionality between rate of assimilation and external concentration (partial pressure) of CO_2.

The case seems most clear for water plants, where a direct proportionality has been demonstrated by Blackman and Smith, 1911 (2), for Elodea. The experiments of Brown and Escombe, 1902 (4), with land leaves, at concentrations of CO_2 up to seven times that of ordinary air, point in the same direction, and although the results of later workers with land leaves (Boysen-Jensen, 1918 (3), and Lundegårdh, 1921 (7)), do not in some other respects conform to the simple type shown by the Elodea results, yet, so far as the direct proportionality between CO_2 concentration and apparent assimilation is concerned, the position is rather strengthened than otherwise.

Warburg, 1919 (9), and Harder, 1921 (6), however, working on water plants, failed to find a direct proportionality between CO_2 concentration and assimilation; moreover, the interaction between light intensity and CO_2 concentration shown by their results, and by those of Boysen-Jensen and Lundegårdh, presents a problem which cannot be analysed in the simple terms which were adequate to the Elodea experiments of Blackman and Smith.

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* This and the succeeding communication form part of a thesis accepted for the Ph.D. Degree, Cambridge University, 1925. The work was begun at the suggestion of Dr. F. F. Blackman and carried out in his laboratory at the Botany School, Cambridge. The writer is greatly indebted to Dr. Blackman for his continual stimulating interest and helpful criticism.

The experimental work described here was begun in order to see how far the analysis in terms of "limiting factors" could be applied to land leaves. In particular it was hoped that, by taking continuous records of the assimilation over a period of 12 to 24 hours, and by changing the external conditions during the period of experiment, the relationships of the various controlling factors might become more clear.

(b) *Experimental Methods.*

1. *Material.*—Most of the work has been done with leaves of Cherry Laurel (*Prunus laurocerasus*, var. *rotundifolia*). The leaves used were those of the second or third node on branches of the current year on the same part of the bush. Two bushes were drawn upon—a few early experiments being carried out with leaves from a bush near the Botany School (these will be called "Botany School leaves"), the rest of the experiments with leaves from a bush in the Botanic Gardens.* The leaves were brought to the laboratory, with their stalks in water in a tin, and were then kept, with their stalks in water, covered with a bell-jar in the diffuse light of a roof greenhouse, at an approximately steady temperature until they were required.

The leaves were weighed just before and just after the experiment. They varied in size between 42 sq. cm. and 57 sq. cm. in area, the mean being 49·1 sq. cm. The areas were determined by means of a planimeter.

2. *Apparatus.*—The apparatus used is that designed by Dr. F. F. Blackman and described by Miss Matthaei (8). Briefly, a pair of balanced aspirators maintain a steady double current of air, which, by their balanced action, is equally divided between the leaf chamber and a control chamber. The air as it enters the apparatus is enriched with CO_2, by means of weak acid (HCl), dropping on to marble contained in a vertical tube through which the air passes.† Following this vertical tube comes the Y tube, at which the enriched air is divided into the two halves of the apparatus. The CO_2 remaining in the two air currents, after passing through the leaf chamber and the control chamber respectively, is estimated by absorption (by baryta) in pairs of Pettenkofer tubes. The difference between the amount absorbed from the control current and that absorbed from the plant current is a measure of the apparent assimilation of the leaf. Blank experiments, to test the equal division of the enriched

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* In any experiment the source of the leaf is indicated by the letters B.S. (Botany School) or B.G. (Botanic Garden) following the number of the experiment.

† Variation in CO_2 concentration of the air current is obtained by varying the rate of dropping of the acid.

air between leaf chamber and control chamber, showed the division accurate to within 1 per cent.

A clockwork arrangement enables successive one-hourly, two-hourly, or three-hourly estimations to be made in successive pairs of Pettenkofer tubes.

The technique of titration was that employed by Miss Matthaei (*loc. cit.*) and the strengths of baryta and acid generally used were, approximately, baryta N/5; acid (HCl) N/10.

The aspirator rate was approximately 1500 c.c. per hour, and was measured at regular intervals during an experiment. The necessary corrections having been made, the CO_2 concentration in the supplied air was calculated as the number of cubic centimetres of CO_2 (at 0° and 760 mm.) contained in 100 c.c. of air at 0° C., and at the pressure of air in the leaf chamber. This gives the partial pressure of the CO_2 as percentage of an atmosphere.

The *mean concentration of* CO_2 in the leaf chamber is calculated as

$$\frac{C_1 - C_2}{\log_e (C_1 \div C_2)},$$

where C_1 is the concentration in the air entering, and C_2 that in the air leaving, the leaf chamber.

The values for apparent assimilation are given as cubic centimetres of CO_2 (at 0° and 760 mm.) per 50 sq. cm. of leaf surface (one surface only) per hour.

3. *Leaf Chamber.*—In the earlier experiments a large leaf chamber was employed, consisting of an oval rim of iron, on to which were fastened, with luting wax, two glass plates. One of these remained permanently fastened down, the other could be removed for the insertion of the leaf and afterwards re-waxed. The dimensions of this chamber were 22 cm. long by 16 cm. broad by 1·8 cm. deep, and the volume was 400 c.c. Inlet and exit tubes were at opposite sides of the chamber, and a thermometer projected into the chamber from the top. The leaf stalk rested in a small glass cup, at the bottom of the chamber, holding 2 to 5 c.c. of water.

A smaller chamber, used in most of the experiments, was of similar structure, but approximated more closely to the shape of a leaf. The dimensions were 15·5 cm. long by 7·5 cm. broad by 1 cm. deep, and the internal volume about 80 c.c. The air inlet was near the base, on one side; the exit was at the top. The metal rim of the chamber was too narrow to permit the introduction of a thermometer, but, from the experiments with the large chamber (and subsequent experiments with the small chamber), the excess of the air temperature in the chamber over that registered by a thermometer just outside was known for each intensity of illumination employed. When using the small chamber, therefore, a thermometer was hung just outside the leaf chamber.

The leaf stalk dipped into 1 to 2 c.c. of water contained in the bottom of the chamber.

4. *The Source of Light* was a Keith high-pressure triple incandescent gas burner—the three mantles being arranged on a triangular ground plan, a side of the triangle being towards the leaf. Thus the third mantle, behind, filled up the space between the two front ones and the whole gave a source of light, viewed from the leaf chamber side, 10 cm. high by 7 cm. broad. (The dimensions of the leaves were 10 to 12 cm. long by 5 to 6 cm. broad.) Between the light source and the leaf chamber was a thick screen of flowing water to cut down the heat radiation.

Two intensities of light were employed: (*a*) "*High*" *light* with the front of the leaf chamber 8·4 cm. distant from the front of the mantles; (*b*) "*low*" *light* with the distance = 17·5 cm., the leaf chambers being held vertically in stands at the required distance from the light. The distance between the leaf surface and the front of the mantles is almost exactly doubled at the lower light intensity.

Measured by means of a Callendar recorder at distances from the mantles ranging from 12 cm. (the nearest that could be obtained) and 25 cm., the intensity of the total radiation of this large source of light is found to approximate more nearly to an inverse linear relation with distance than to an inverse square relation. The photosynthetic values of the two light intensities are approximately in the ratio of 2·4 to 1, as may be seen from the subjoined table, which gives the "light limited" values of assimilation obtained with these two light intensities during the summer of 1920.

In the subsequent account of the experimental work the two light intensities will be referred to simply as "high" light and "low" light respectively.

Table I.

1920.	Light at	Apparent Assimilation.	Respiration.	Real Assimilation.*
	cm.	c.c.	c.c.	c.c.
September 6–7	8·4	5·8	0·4	6·2
September 9	17·5	2·295	0·3	2·595

* *Note.*—(*a*) The relative magnitude of the light intensities employed may be judged by the fact that the photosynthetic value of 0·28 sunlight for Cherry Laurel leaves is 5·8 c.c. per 50 cm.^{2} per hour. (Blackman and Matthaei, 1905 (1).)

(*b*) Respiration figures in the above table are calculated from Matthaei's table (8).

Respiration was measured in a few experiments only, when the figures were found to agree closely with those of Miss Matthaei (8). Attention was concentrated rather on the relation between the external CO_2 concentration and the uptake of CO_2 by the leaf—apparent assimilation—so that, unless otherwise specified, all references to assimilation are references to *apparent assimilation.*

The justification for this procedure will appear in the paper itself. It is sufficient to state here that with either of the two light intensities employed no measurable amount of CO_2 was given off by the leaf when an air current free from CO_2 was drawn over it, *i.e.*, at zero external CO_2 concentration, the apparent assimilation is approximately zero. Thus, for that region of the CO_2 concentration-assimilation curve in which approximate linearity prevails, the ratio of apparent assimilation to external CO_2 concentration will be approximately constant, while the relation between external CO_2 concentration and real assimilation will be less direct.

SECTION II.—THE DAILY MARCH OF ASSIMILATION UNDER "LIMITING" CONDITIONS OF CO_2 SUPPLY AT CONSTANT LIGHT INTENSITY.

(*a*) *Experiments on the Daily March of Assimilation, July and August,* 1920.

Experiments were begun in July, 1920, using the "high" light and a concentration of CO_2 considerably less than that known to be required for the maximal assimilation at the "high" light intensity. That is, the conditions were so arranged that the CO_2 supply should be, in Blackman's sense, "limiting."

An early experiment, Experiment III, on a "Botany School" leaf, is shown graphically in fig. 1 (p. 472), in which apparent assimilation is indicated by the heavy line and mean CO_2 per cent. by the broken line. The leaf was picked at 9 a.m. and set up in the leaf chamber *as soon as possible after picking.* The chamber was fixed at 8·4 cm. from the light source and the air currents started. After a preliminary period of 1½ hours, 2-hourly readings of the apparent assimilation were begun, and continued until 9 a.m. the following morning. The assimilation shows a well-marked depression, "*a first minimum,*" at the second reading, followed by a rise to a value higher than the initial value, and that succeeded by a steady and deep depression culminating before midnight. In the morning the assimilation rises again and approaches the initial values in magnitude.

It was found impossible to maintain the percentage of CO_2 supplied to the leaf quite constant. But, even if this were possible, the mean concentration

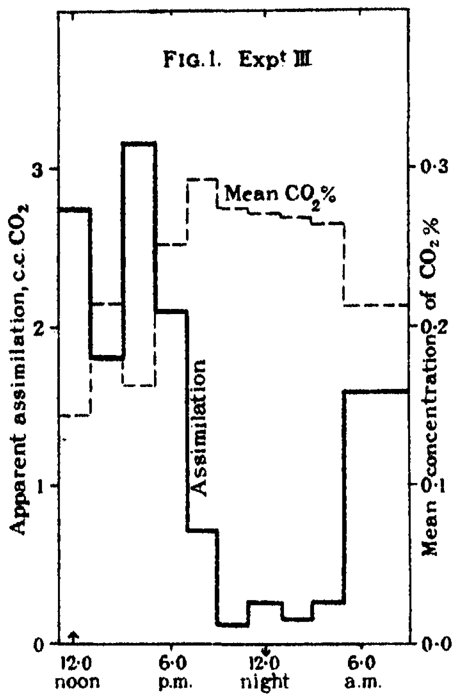
FIG. 1. Expt III

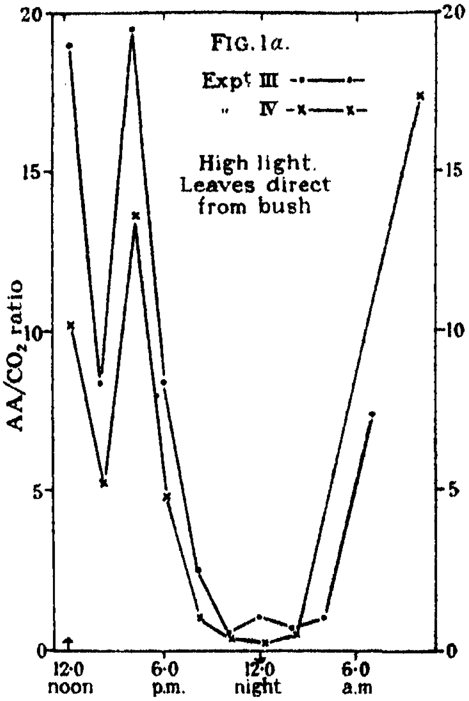
FIG. 1a.

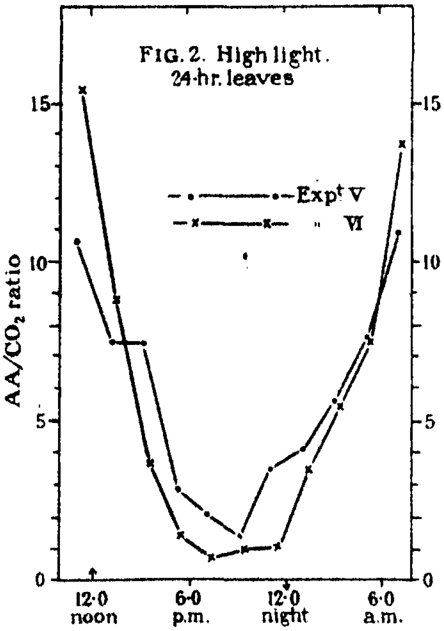
FIG. 2. High light. 24-hr. leaves

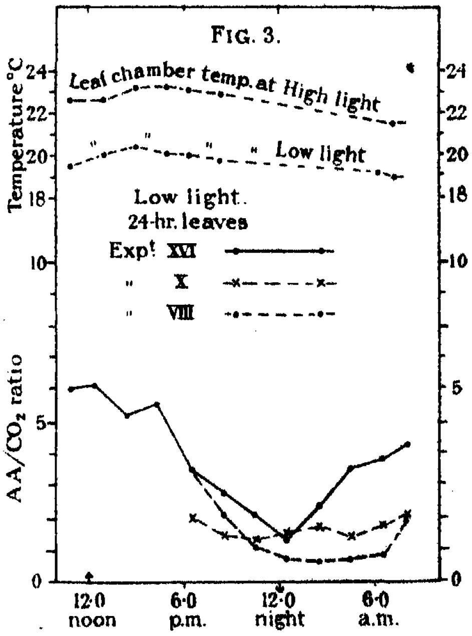
FIG. 3.

FIGS. 1, 1A, 2 and 3.—March of apparent assimilation in Cherry Laurel leaves, summer 1920.

FIG. 1.—Experiment III, July 29–30. A Botany School leaf, direct from the bush. "High" light; average CO_2 = 0·236 per cent.; temperature 21–23° C.

FIG. 1A.—Shows the march of the ratio between apparent assimilation and mean external CO_2 concentration (A.A./CO_2 ratio) for Experiment III, and for a similar experiment, Experiment IV, on a Botanic Garden leaf.

Experiment IV.—July 31–August 1. "High" light; average CO_2 = 0·237 per cent.; temperature 20·8–22·8° C.

FIGS. 2 and 3.—Show experiments in which the leaf was kept 24 hours in diffuse light before being set up—"24-hour leaves."

FIG. 2.—"High" light.

Experiment V, B.S., August 25–26. Average CO_2 = 0·265 per cent.; temperature 22·2–23·8° C.

Experiment VI, B.G., August 26–27. Average CO_2 = 0·278 per cent.; temperature 20·7–23·0° C.

FIG. 3.—"Low" light and three different CO_2 concentrations.

Experiment XVI, B.S., September 13–14. Average CO_2 = 0·315 per cent.; temperature 17·8–20·5° C.

Experiment X, B.S., September 3–4. Average CO_2 = 1·108 per cent.; temperature 20·4–22° C.

Experiment VIII, B.S., August 30–31. Average CO_2 = 0·618 per cent.; temperature 17·6–19·2° C.

In the upper part of fig. 3 is shown the average leaf-chamber temperature for these three experiments at "low" light and also the average temperature for the corresponding experiments at "high" light (*cf.* fig. 2).

of CO_2 in the leaf chamber must alter, with the enormous variation in the amounts of CO_2 removed from the current by the leaf in different 2-hour periods. As will be seen from fig. 1, the mean CO_2 per cent. value varies considerably on either side of its average value of 0·236 per cent., the major variations being clearly determined by the variations in assimilation rate. A better picture of the march of assimilation may therefore be obtained by calculating, for each reading, the ratio between the apparent assimilation and the mean CO_2 concentration. This method of representing the march of assimilation will be generally employed and the values obtained will be referred to as A.A./CO_2 ratios.* The A.A./CO_2 ratios for Experiment III, and for a similar experiment (Experiment IV), two days later, on a Botanic Garden leaf are shown in fig. 1A (p. 472). The general march of assimilation is strikingly parallel in the two cases, though there are differences in the level attained.

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* Alternatively the values calculated may be regarded as values of apparent assimilation corrected to a standard mean CO_2 concentration of 1 per cent. (Lundegårdh has corrected his assimilation values to 0·03 per cent. mean CO_2). While, under the conditions stated, *i.e.*, actual assimilation well below the light-limited value, it is probable that the values will approximate very closely to those which would have been found had the mean CO_2 been maintained constant, yet it seems best to emphasise the provisional nature of the correction by using the term A.A./CO_2 ratio, which describes the process by which the adjustment is made. The method will be used merely as a tool for the preliminary analysis of the phenomena.

As already noted, the light intensity was maintained constant. Variation in air temperature of the leaf chamber was small (range 20·8° to 23°); in general the temperature slowly increases from time of setting up until 8 p.m. or 9 p.m., and then slowly falls off till 8 a.m. or 9 a.m. (compare air-temperature curves in fig. 3).

24-Hour Leaves.—The results shown in fig. 1A are characteristic of leaves picked at this season of the year, and set up as soon as possible after picking. When leaves are picked at the same hour in the morning (9 a.m. to 10 a.m.) but kept 24 hours, their stalks in water, under a bell-jar in diffuse light, before setting up, the subsequent march of assimilation is somewhat simpler.*

The results of the first two experiments with "24-hour leaves" are shown in fig. 2. Only the A.A./CO_2 ratios are given, the average CO_2 per cent.† being very nearly the same for both experiments. (Experiment V, B.S., $CO_2 = 0 \cdot 265$ per cent.; Experiment VI, B.G., $CO_2 = 0 \cdot 278$ per cent.).

The march of assimilation is now more regular; the first minimum has disappeared, but the very great noctural depression and the subsequent morning recovery are as marked as before. These four experiments—III, IV, V, VI—were carried out at "high" light intensity, and the highest assimilation rate observed (3·27 c.c. CO_2 per 50 sq. cm. per hour) is only a little over half the maximal assimilation of 5·8 c.c. CO_2 possible at that light intensity at this season. Hence the belief that the supply of CO_2 is "limiting" the rate appears justified.

(*b*) *Experiments on the Nature of the Nocturnal Depression in Assimilation Rate.*

The elucidation of the nature of this nocturnal depression was the object of the next experiments. Fig. 3 (Experiments VIII, X and XVI, with light at 17·5 cm.) shows that it occurs at the "low" light intensity just as at the "high." It cannot be explained as due to a fall in temperature during the afternoon, since, up to 8 p.m. to 9 p.m., the temperature either hardly alters at all or rises slightly, while by this time the maximum depression in assimilation is in some cases already reached * (figs. 1A and 3).

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* All subsequent experiments except where otherwise noted are on 24-hour leaves.

† The value for average CO_2 concentration given in connection with the figures for each experiment is the average of all the values for mean CO_2 per cent. during the experiment. For periods of high assimilation rate the mean CO_2 will be somewhat below the average, and for periods of low assimilation rate somewhat above the average (*cf.* fig. 1).

Since there was, as indicated above, reason to believe that the CO_2 supply was "limiting" during the experiments, the assumption was made that the depression was due to an increased resistance to diffusion of CO_2 into the leaf. Certain consequences of this assumption were tested by means of a series of experiments at a constant light intensity with increasing concentrations of CO_2. In order to avoid very high concentrations of CO_2, the "low" light intensity was employed for these experiments.

Assuming that the diurnal march of this alteration in the resistance offered by the leaf to inward diffusion of CO_2 remains constant throughout the series, the ideal result of such a series of experiments would be a set of curves in which, as the CO_2 concentration increases, the depressed night values are "pushed up," until they *all* stop at the limit set by the light intensity employed.

Fig. 4 (p. 476) (Experiments XVI, VIII, X and XIV) shows that this result is approximately realised. (1) Experiment XVI, at 0·315 per cent. CO_2: the fall in assimilation begins at once. (2) Experiment VIII, at 0·617 per cent. CO_2: the characteristic fall is delayed until after the fifth reading. The first five readings show a gradual fall, but may be taken as representing values mainly limited by light intensity. (3) Experiment X, at 1·108 per cent. CO_2: the first reading (for a period of one hour only) is unexpectedly low, but the next four are gradually falling, mainly light limited, values. The night values are "pushed up" as compared with those of Experiments XVI and VII, and the final value is about equal to the initial "light limited" values. As compared with Experiment XVI, Experiment VII shows, in its night values for assimilation (with the exception of the sixth reading), no increase with increased CO_2 supply. That this is to be ascribed to variations between individual leaves in the march and extent of the nocturnal change in resistance to diffusion, is suggested by fig. 3 (p. 472), where are plotted the A.A./CO_2 ratios (for all except the "light limited" assimilation readings) of Experiments XVI, X and VIII. The curve for Experiment X, at 1·1 per cent. CO_2, lies between that for XVI, at 0·315 per cent., and for VIII, at 0·618 per cent. (4) Experiment XIV, at 2·333 per cent. CO_2: in this experiment, owing to the high concentration of CO_2 used, hourly titrations of the Pettenkofer tubes were necessary, and it

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* In addition, experiments carried out by G. E. Briggs and the writer have shown that within the range of light intensities and carbon dioxide concentrations here considered changes in air temperature between 15° and 25° C. have very little effect upon apparent assimilation of Cherry Laurel leaves.

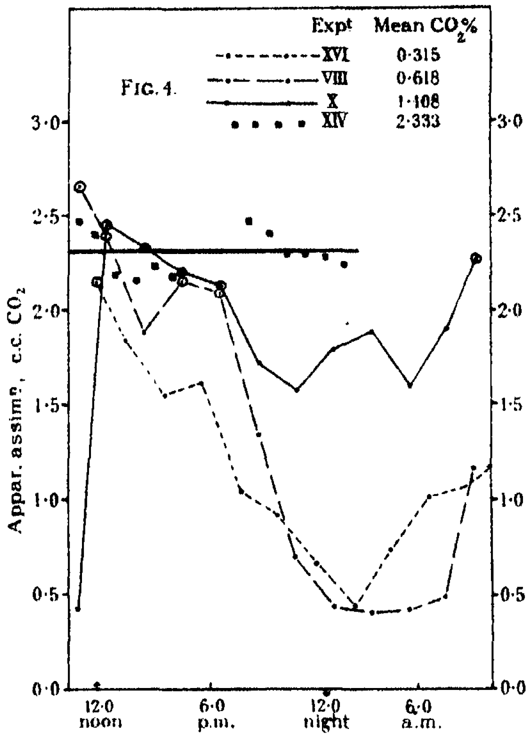


Fig. 4.—The effect of increasing CO_2 concentration on march of apparent assimilation at "low" light intensity. All Botany School leaves.

Experiment XVI, September 13–14. Average CO_2 = 0·315 per cent.; temperature 17·8–20·5° C.

Experiment VIII, August 30–31. Average CO_2 = 0·618 per cent.; temperature 17·6–19·2° C.

Experiment X, September 3–4. Average CO_2 = 1·108 per cent.; temperature 20·4–22° C.

Experiment XIV, September 9–10. Average CO_2 = 2·333 per cent.; temperature 21·4–22° C.

Values of assimilation which are regarded as mainly light limited are distinguished by circles, except in the case of Experiment XIV, where all the values are light limited. The average assimilation value, 2·3 c.c. CO_2, obtained in Experiment XIV, for the "low" light limit, is shown in the figure as the thick horizontal line.

was not possible, therefore, to carry on the experiment beyond 1.30 a.m. At this high concentration, moreover, differences, even less than half per cent., between the two air currents, plant and control, are bound to cause variations of several per cent. in the amounts of assimilation calculated. But, as the figure shows, the observed values fluctuate definitely about a level mean (which is interpreted as the light limit for "low" light intensity) and show no sign of a nocturnal depression. The anticipated result is thus realised, as nearly as could be hoped in an experiment depending upon an analysis of air currents for the estimation of apparent assimilation; and it seems clear that the nocturnal depression of the other experiments (figs. 1 to 4) cannot be ascribed to any depression in the activity of the photosynthetic process itself (nor to any huge increase during the night hours in respiration). It seems fair to describe the nocturnal depression as a phenomenon in the diffusion phase of the process of assimilation.

A more complete experiment at a later date confirms these conclusions, Experiment XLIII, B.G., November 12–13, fig. 5. It will be noted that the average light limited value is higher than before (the average value for the first 14 readings is 2·66 c.c. as against 2·30 c.c. for the experiment in

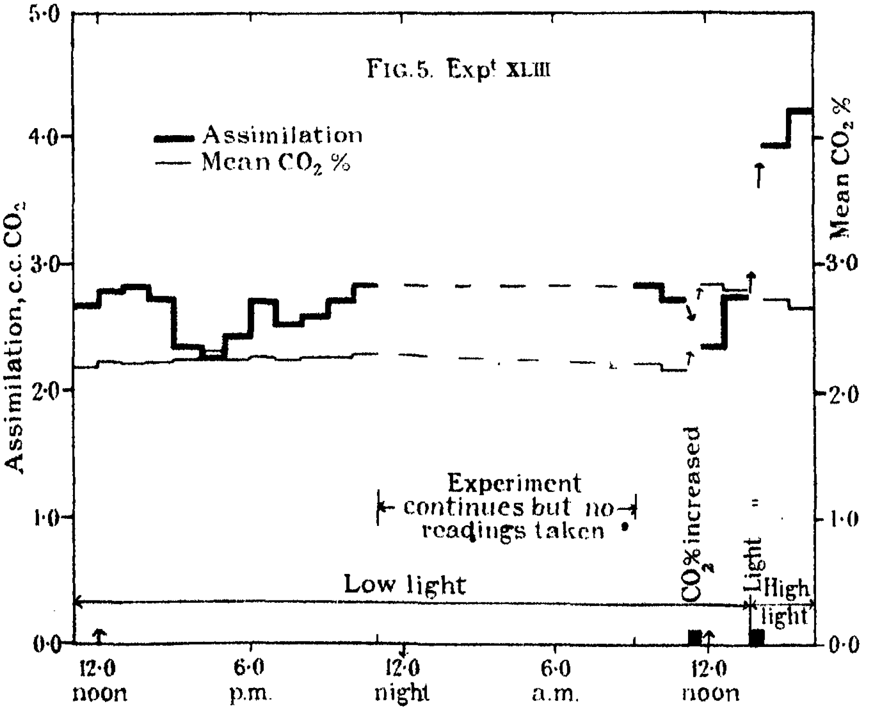


FIG. 5.—March of apparent assimilation at high CO_2 concentration and "low" light intensity.

Experiment XLIII, B.G., November 12–13. Average CO_2 = 2·255 per cent. up to 11.15 a.m. November 13, then increased to 2·82 per cent. Light intensity "low" till 1.45 p.m., November 13, then increased to "high." Temperature 19–21·2° C. Average apparent assimilation at 2·255 per cent. CO_2 = 2·657 c.c. CO_2. Average of two readings at 2·82 per cent. CO_2 = 2·573 c.c. CO_2.

September). This is in accord with Miss Matthaei's observations (8) on the seasonal variations in photosynthetic activity in these leaves, a subject which will be considered more fully in a subsequent paper.

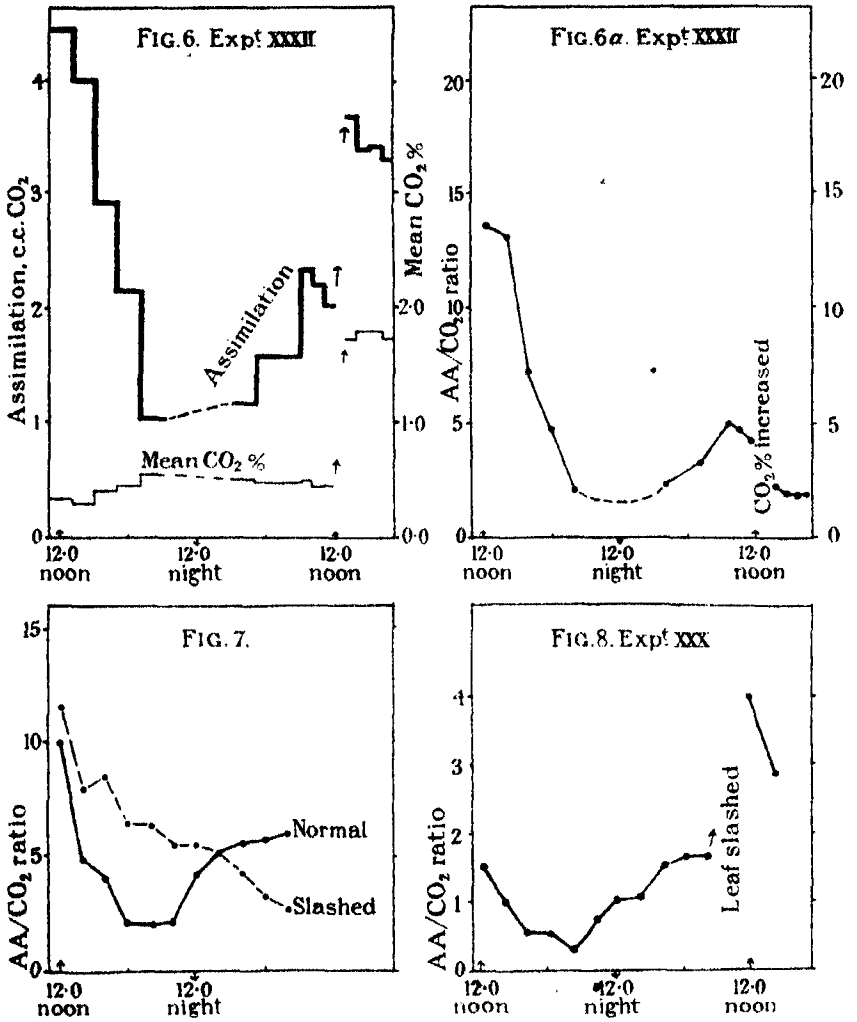
For this experiment the small leaf chamber was used, since it was desired to change the external conditions during the experiment, and a chamber of small volume was necessary in order to avoid long "preliminary" periods before making a reading under the new conditions. With this chamber a preliminary period of half an hour was used; in that time a volume of air 8 to 9 times the volume of the chamber would pass through it.*

The CO_2 per cent. used was high (2·25 per cent.) and hourly readings were necessary; but again the values fluctuate fairly closely about a mean. Up to 11 p.m. no depression in assimilation is shown, and two readings next morning (9.15 a.m.–11.15 a.m.) are but slightly above the previous day's mean. At this point the CO_2 per cent. was increased from 2·18 per cent. to 2·82 per cent. The assimilation values still lie about the same mean. Finally, the chamber was moved to 8·4 cm. distance from the light source. The assimilation rises from a mean of 2·573 c.c. (two readings) to a mean of 4·127 c.c. (two readings). There can be no doubt but that the assimilation for the first 26¾ hours of the experiment is light limited, and that the light limited value is approximately constant during this period.

One further experiment on the results of changing the concentration of CO_2 must be considered, for this reason—that during September and October the form of the A.A./CO_2 ratio curves changed gradually from the typical U form of August to a L form. (The L form may be regarded as a combination of the nocturnal depression characteristic of earlier experiments and a general falling curve.) It was therefore necessary to examine whether here again we were dealing with changes in the resistances in the diffusion phase rather than in the chloroplast mechanism.

A typical experiment is shown in fig. 6, Experiment XXXII, October 18 (p. 479). To economise time on the second morning, the assimilation was not recorded between 9 p.m. and 3 a.m., but the curve is clearly of the L form. After the morning maximum on the second day the CO_2 concentration was increased from 0·475 per cent. to 1·715 per cent. The assimilation rises at once, and the fall with time, which began before the CO_2 per cent. was increased, continues at the higher level. The A.A./CO_2 ratios for this experiment are shown in fig. 6A, and, although the percentage increase in assimilation, when the CO_2 supply was increased, was less than the percentage increase in CO_2 supply,

* The small chamber was used in all subsequent experiments.



Figs. 6, 6a, 7 and 8.—Effect of increasing the CO_2 concentration, and of "slashing" the leaf, upon Apparent Assimilation.

Fig. 6.—Experiment XXXII, B.G., October 18–19. "High" light: average CO_2 = 0·449 per cent., increased at 12.5 a.m., October 19, to 1·752 per cent.; temperature 17·4–19° C. Apparent assimilation shown by heavy lines; mean CO_2 per cent. by light lines.

Fig. 6a.—Shows the march of the A.A./CO_2 ratios for Experiment XXXII (fig. 6).

Fig. 7.—March of assimilation in a "slashed" and an unslashed leaf.

Experiment XXVII, B.G., October 8–9. Leaf "slashed" between the main lateral veins. "High" light: average CO_2 = 0·4213 per cent.; temperature 22–23·5° C.

Experiment XXVIII, B.G., October 11–12. Leaf not "slashed." "High" light: average CO_2 = 0·3471 per cent.; temperature 19·5–20·5° C.

Fig. 8.—Effect of "slashing" a leaf during an experiment. Experiment XXX, B.G., October 13–14. "Low" light: average CO_2 = 0·368 per cent. Leaf removed at 7.11 a.m., October 14, and "slashed" between main veins; set up again 9.20 a.m. Readings recommence 10.55 a.m.

yet the A.A./CO_2 ratios for these readings occupy roughly the position to be expected from the earlier readings at the lower CO_2 concentration. Three other experiments—XXXI, XXXIII and XXXV, October 15–26—gave confirmatory results.

"*Slashed*" *Leaves*.—An effect upon apparent assimilation, similar to that produced by increasing the CO_2 concentration, can be obtained by making cuts in the leaf. A typical experiment (Experiment XXX) is shown in fig. 8 (p. 479). The CO_2 concentration was not increased during the experiment; hence, only the A.A./CO_2 ratios are plotted. These are low,* but show the usual nocturnal depression and a gradual morning rise. At 9.11 a.m. the leaf was taken out and long cuts were made between the main lateral veins on each side. The ratio rises from 1·735 before the "slashing" to 3·97 after the "slashing," but the next value shows a decline to 2·84. Other similar experiments confirmed this result of "slashing" upon the apparent assimilation of the "CO_2 limited" leaf, and it was thought that with "slashed" leaves it might be possible to eliminate the nocturnal depression and other depressions in the A.A./CO_2 ratios, and so obtain steady values of assimilation which could be used for a CO_2 concentration-assimilation curve. With one exception, however, Experiment XX, (at "low" light intensity), these leaves all lost weight by drying during the experiments (a loss of from 10 to 20 per cent. of their original weight), and the assimilation values showed a steady decline. Experiment XX in which the assimilation values did not decline, was at high CO_2 concentration (2·5 per cent.), and gave values of assimilation fluctuating about a mean of 2·208, which is approximately the light limited value for the "low" light intensity at this season. This result is evidence that "slashing" does not operate by stimulating the photosynthetic process itself, since the light limited assimilation value is the same as for normal leaves.

A typical experiment with a "slashed" leaf at "limiting" concentrations of CO_2 is shown in fig. 7 (Experiment XXVII, October 9–10, 1920 (p. 479)), and may be compared with an experiment on an unslashed leaf two days later (Experiment XXVIII, October 11–12).† The nocturnal depression is eliminated in the "slashed" leaf, the night values being considerably higher than for the unslashed leaf, but the steady fall brings them below the values of the normal leaf on the morning of the second day. It seems reasonable to attribute this behaviour on the part of slashed leaves to a decreased water content in the cell walls of the leaf, which would markedly retard the passage of CO_2 through them. By the courtesy of Mr. P. Parija I am able to quote some experimental results obtained by him in this laboratory (Cambridge Botany School) bearing on this point.

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* The "low" light intensity was employed for this experiment.

† The average external CO_2 concentration for the "slashed" leaf is higher than that for the normal leaf, 0·421 per cent. against 0·347 per cent. All the values of apparent assimilation are, however, far below the light limited value, and comparison between the two curves is not invalidated by the difference in external CO_2 concentration.

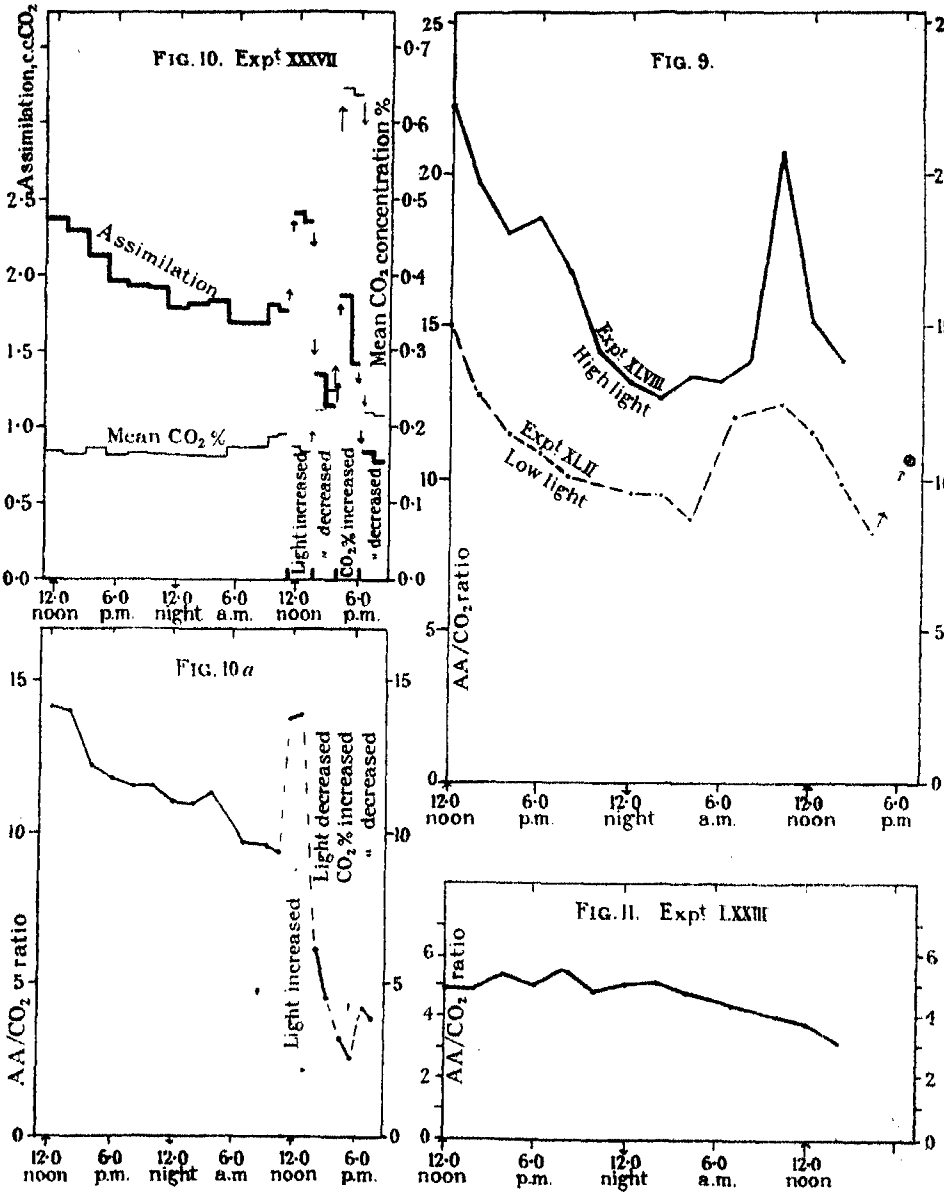
Table II. - Relation between Moisture Content of a Gelatine Film and its Permeability for CO_2. (Data by Mr. P. Parija, Botany School, Cambridge.)

Percentage of water in gelatine film.	Relative rate of diffusion of CO_2 through the film.
Per cent.	
23	1
29	3
44	20
66	56
77	144

It is not intended to press the analogy between a gelatine film and the wet cell wall, but the results are undoubtedly suggestive.

(*c*) *The Effect of Light Intensity upon the " CO_2 limited " Assimilation Curves.*

A change towards the end of September in the form of the diurnal curves for the A.A./CO_2 ratio has already been noted. Towards the end of October the curves change again in form. As a rule, however, good agreement is shown between curves of about the same date. We may, therefore, best compare experiments at " high " light and " low " light by grouping experiments of about the same date. Fig. 2 (" high " light) and fig. 3 (" low " light) afford a comparison during August and September; for the early part of October figs. 6A and 7, at " high " light, may be compared with fig. 8, at " low " light. (The vertical scale of fig. 8 is three times that of figs. 6A and 7.) The general level of the curves at " low " light is markedly below that of the curves at " high " light. A similar comparison for November will be found in fig. 9 (p. 482). The nocturnal depression, though quite definite at both " high " and " low " light intensity, is now very much less marked than before, and the general level of the curves, especially in the case of the " low " light experiment, has very greatly increased. The difference between the results at " low " and at " high " light is relatively less than before, but the " high " light curve is still well above that at " low " light.



Figs. 9, 10, 10a and 11.

Fig. 9.—March of assimilation, November, 1920, at "high" and at "low" light intensity.

Experiment XLVIII, B.G., November 29–30. "High" light: average CO_2 = 0·155 per cent.; temperature 19·8–21·4° C.

Experiment XLII, B.G., November 10–11. "Low" light: average CO_2 = 0·127 per cent.; temperature 19–21·1° C. At 5.0 p.m., November 11, the light intensity was increased to the "high" level, so that the last reading for this experiment is for a period of "high" light.

FIGS. 10, 10A and 11—(continued).

FIG. 10.—Effect of change in light intensity on apparent assimilation at low (*i.e.*, "limiting") concentrations of CO_2. Experiment XXXVII, B.G., October 29–30. Light "low" till 11.0 a.m., October 30; "high" 11.0 a.m. to 1.20 p.m.; then "low" for rest of experiment. Average CO_2 = 0·178 per cent. till 3.45 p.m., October 30; from 3.45 p.m. to 6.5 p.m. = 0·644 per cent.; from 6.5 p.m. to 8.25 p.m. = 0·216 per cent. Temperature 16·4–19·2° C.

FIG. 10A.—A.A./CO_2 ratios for Experiment XXXVII (fig. 10). The points at which some change was made in the external conditions are shown by the broken lines in the curve.

FIG. 11.—March of assimilation in a Hydrangea leaf. Experiment LXXIII, April 21–22. Leaf of Hydrangea sp. "High" light: average CO_2 = 0·480 per cent. Temperature 20–21·6° C.

To confirm this general result, namely, the improvement in the A.A./CO_2 ratios with increased illumination, a number of experiments were carried out in which a change in light intensity was made during the course of the experiment. A typical experiment is represented in figs. 10 and 10A (Experiment XXXVII, October 29–30). The experiment began at 10 a.m. using the "low" light intensity and a low concentration of CO_2 (about 0·17 per cent.). Apparent assimilation begins at a rate of 2·365 c.c. (the light limited value is 2·6 c.c. for this season) and shows the usual nocturnal fall up to midnight. There is not, however, any morning recovery, and by 11 a.m. assimilation has fallen to 1·766 c.c. At this point the light intensity was increased to the "high" level: the assimilation rises to values of 2·4 c.c. and 2·35 c.c., although the mean CO_2 per cent. falls slightly. On reducing the light intensity to its original level, the assimilation falls to 1·36 c.c. and 1·13 c.c., although the CO_2 per cent. has increased somewhat. Next, keeping the light constant, the CO_2 supply is increased to 0·644 per cent. The assimilation rises, though the fall with time, which began in the previous period at low CO_2 per cent., continues. Finally, by reducing the CO_2 supply to about its original level, there is produced a further fall in the assimilation.

The A.A./CO_2 ratios for this experiment are given in fig. 10A, which shows clearly the very marked effect of the increase in light intensity. It will be noted also that, although, as is clear from fig. 10, the increase in assimilation brought about by increasing the CO_2 concentration is not proportional to the increase in CO_2, yet the A.A./CO_2 ratios for the period of high CO_2 per cent. fall well into line with those for the preceding period at low CO_2 per cent. There is, however, some recovery in the succeeding period at low CO_2 per cent. The general result is quite clear that, at "limiting concentrations" of CO_2, assimilation can be increased either by increasing the CO_2 concentration or by increasing the light intensity. Thus we have here not one external controlling factor—CO_2—but two, namely, light and CO_2.

A summary of certain aspects of the results which have now been considered will be found in Table III, in which are given averages calculated for the first 22 hours of the experiments (approximately 11 a.m. to 9 a.m.). In some experiments a few readings were omitted (*e.g.*, Experiment XXXII, fig. 6); in such cases the mean value of the two neighbouring readings is taken to supply the gap in the diurnal series.

Table III.—Assimilation Values at "Limiting" Concentrations of CO_2; August to November, 1920. 22-Hour Averages.

Date.	Leaf.	Mean CO_2 per cent.	Apparent Assimilation.	A.A. as per cent. of light limited value.	A.A./CO_2 ratio.	
					"Low" light.	"High" light.
August 25–26	B.S.	0·265	1·436	24·8		5·79
August 26–27	B.G.	0·278	1·370	23·6		5·69
August 31–September 1	B.G.	0·610	0·722	31·4	1·170	
September 13–14	B.S.	0·315	1·157	50·3	3·300	
October 11–12	B.G.	0·347	1·545	26·7		4·71
October 13–14	B.G.	0·367	0·376	16·3	1·065	
October 18–19	B.G.	0·449	2·022	35·0		5·04
October 29–30	B.G.	0·178	1·945	74·8	11·66	(+4·4=16·06)
November 10–11	B.G.	0·127	1·277	49·2	10·0	(+2·3=12·3)
November 29–30	B.G.	0·155	2·545	38·9		15·98

In the table, the column following that for apparent assimilation shows what percentage the apparent assimilation is of the maximum possible at the light intensity employed. For the experiments up to October 19, the light limits are taken as: "high" light, 5·8 c.c.; "low," 2·3 c.c. After October 19, the limits are: "high," 6·55 c.c.; "low," 2·6 c.c. In two experiments at "low" light, October 29–30 and November 10–11, there were short periods of "high" light at the end of the experiments. The increase shown by the A.A./CO_2 ratio for these periods over the ratio for the preceding period at "low" light is given in this table, and, added to the 22-hour average value for the "low" light, forms an estimate of the 22-hour average for "high" light at that season. The estimates, it will be seen, agree well with the 22-hour average obtained for "high" light on November 29–30.

It will be noted that, owing to the very great increase, especially for the "low" light, in the A.A./CO_2 ratios at the end of October, very much lower concentrations of CO_2 had to be employed in order to keep the apparent assimilation well below the light limited value, *i.e.*, to maintain conditions in which CO_2 concentration should be "limiting." It is interesting to observe that, at "low" light intensity, the highest average value of the A.A./CO_2 ratio, 11·66, is associated with the highest average value of the apparent assimilation, 1·945 c.c. (or 74·8 per cent. of the maximum), while the lowest A.A./CO_2 value, 1·065, is associated with the lowest value of apparent assimilation, 0·376 c.c. (or 16·3 per cent. of the maximum). The same is roughly true for the experiments at "high" light.

In making use, for a provisional analysis of the data considered in this paper, of the A.A./CO_2 ratio, it was recognised that, without any change in any external or internal factor this ratio might, as CO_2 supply was increased, begin to fall off *before* the light limit was reached, so that absolute constancy of the ratio would not be maintained. It is quite clear, however, both in the diurnal and the seasonal changes shown by the assimilation of these leaves, that this possibility can for the moment be left out of account, since we here have a decreased A.A./CO_2 ratio associated, not with an increase of the assimilation up to near the light limit, but with an actual decrease of the assimilation at somewhat enhanced CO_2 supply. Some powerful factor with a pronounced diurnal and seasonal rhythm is obviously concerned in the phenomena.

(*d*) *Provisional Conclusions as to the Nature of the Diurnal and Seasonal Changes in the Ratio of Assimilation Rate to* CO_2 *Supply.*

The evidence so far presented has shown that the nocturnal depression in assimilation here described is not due to any decrease in the efficiency of the photosynthetic mechanism itself, nor to diurnal changes in respiration rate, but is probably located in the diffusion phase of the process, and represents an increased resistance to the diffusion of CO_2 towards the chloroplasts. Without changing the CO_2 supply to any leaf, the assimilation can be increased, either by "slashing" the leaf or by increasing the light intensity. Further, for a given mean CO_2 concentration, the diurnal assimilation curve is consistently higher at the higher light intensity.

These facts point most strongly to the conclusion that the observed fluctuations in assimilation are mainly due to changes in the degree of stomatal opening. The rhythm of nocturnal depression shown so well in August, October and November (figs, 3, 7, 9) is paralleled by the rhythm of nocturnal stomatal closure described for this plant by Darwin (5), though it is surprising that, with a constant light intensity of three high-pressure incandescent burners at such close range, the effect should be so marked. The August experiments, which show the rhythm most clearly, agree with Darwin's conclusions for Cherry Laurel in placing the maximum effect of closure before midnight.

Having come to this conclusion it was of interest to examine the diurnal behaviour of a leaf which is reputed to possess "immobile" stomata. This was not possible until April 21, 1921, when some plants of Hydrangea sp. were available. It is not maintained that the stomata of Hydrangea show absolutely no movement, but it was expected that at constant light intensity the stomatal opening should remain reasonably constant.*

The march of apparent assimilation at constant light intensity, and a "limiting" CO_2 concentration, in an experiment with a leaf of this species, is shown in fig. 11 (p. 482). Up to 3 a.m., by which time the nocturnal depression shown by Cherry Laurel leaves has usually passed its maximum, the assimilation fluctuates about a level value. Subsequently a slow fall sets in, but there is no approximation to the type of behaviour shown by the normal Cherry Laurel leaves. The only parallel is the "slashed" Cherry Laurel leaf of Experiment XXVII (fig. 7).

So far as it goes this result is in favour of the stomatal explanation of the diurnal rhythm of assimilation in Cherry Laurel. The stomatal explanation also accords well with the observed effect of light intensity upon assimilation at "limiting" concentrations of CO_2 and may obviously be extended to cover the seasonal rhythm, since this may be interpreted as a modification of the range or pitch of the diurnal rhythm of stomatal opening.

That the stoma should, in spite of a high constant light intensity, show such a pronounced rhythm of opening and closing, is in itself a phenomenon of very great interest. That the effect of change in stomatal opening upon assimilation should be so marked, is no less interesting, while, from the point of view of an analysis of the relation between light, carbon dioxide and assimilation rate, it is clearly of fundamental importance. The problem of a heterogeneous reaction having a diffusion phase, the "speed" of which may be slow compared with that of the reactions at the "active surface," is raised in an acute form. Also when, as here, we have to deal with a factor, light intensity, which may affect the "speed" of the diffusion phase, as well as that of the photochemical phase of the process, the problem becomes very complex indeed.

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* That this is the case seems probable from measurements of the porometer rates of leaves of this species under constant light intensity. The rates fluctuate very slightly about a mean for a period of at least 14 hours. The investigation of porometer rates is, however, the subject of the subsequent paper.

In a subsequent paper an attempt will be made, by means of observations on porometer rates as well as assimilation rates, to carry the analysis of the problem a stage farther.

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Experimental Researches on Vegetable Assimilation and Respiration. XVIII.—*The Relation between Stomatal Opening and Assimilation.—A Critical Study of Assimilation Rates and Porometer Rates in Leaves of Cherry Laurel.*

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(Communicated by Dr. F. F. Blackman, F.R.S.—Received October 29, 1927.)

SECTION I.

In a previous communication it was shown that there is, in Cherry Laurel leaves, at "limiting" concentrations of CO_2, a marked diurnal rhythm of apparent assimilation. In spite of the maintenance of a constant high light intensity, the assimilation rate falls to very low values during the night but rises again in the morning. At high concentrations of CO_2, such that light, instead of CO_2, is limiting the rate, there is no diurnal rhythm, the light limited value of assimilation being steadily maintained for more than 24 hours. There is, in addition to the diurnal rhythm, a seasonal rhythm of assimilation —the pitch or level of the diurnal assimilation curves being higher in November than in July, August and September. Also the level of assimilation, for a given "limiting" concentration of CO_2, is higher at high light intensity than at low. It was suggested that these variations in assimilation were largely due to variations in stomatal opening.

The work on porometer rates and assimilation rates described in the present paper was undertaken in order to test this hypothesis and to explore, as far as was possible by such means, the relation between stomatal opening and assimilation rate.

(*a*) *Experimental Methods.*

The methods used for measuring assimilation rate and for calculating the results are fully described in the previous communication (12). The additional technique, adopted for the purpose of estimating changes in stomatal aperture of a leaf at the same time as the assimilation was being measured, is described below.

Darwin's porometer method (6) was adopted as the most convenient and sensitive method of estimating changes in stomatal aperture, and the apparatus finally used is shown in fig. 1. The *porometer chamber*—a glass tube, flanged at one end—is inserted into the leaf chamber through a hole in the back glass plate slightly to one side of the middle line (so as to avoid the mid-rib of the leaf). It is supported by an outer glass tube, or sleeve, in which it just slides freely, and which is fastened securely with shellac to the back plate of the chamber over the hole. Contact with the leaf is made, as in Darwin's experiments, by means of a perforated disc of 30 to 40 per cent. gelatine, the usual dimensions of which were: outer diameter, 2·3 cm.; inner, 0·9 cm.

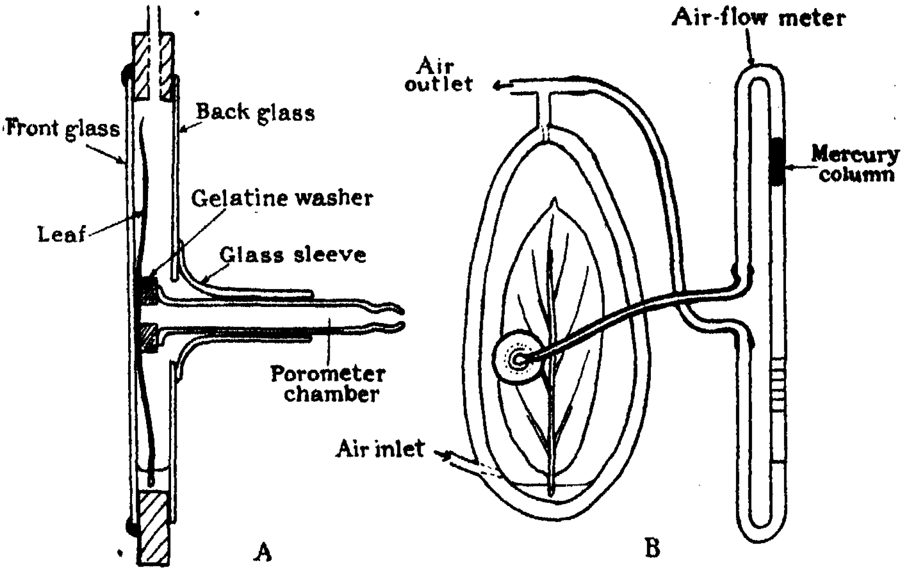


FIG. 1.—Combined assimilation chamber and porometer for simultaneous investigation of apparent assimilation and stomatal behaviour. A. Section of leaf chamber passing through porometer chamber. B. Back view of leaf chamber showing also air-flow meter attached by pressure tubing to porometer and to leaf chamber.

The leaf is put into the leaf chamber, the front glass waxed on, and the porometer chamber pushed home so as just to make air-tight contact between leaf and gelatine and between gelatine and porometer chamber. This involves only a slight pressure on the leaf. The porometer chamber is held in this position while paraffin wax (60° m.p.) is melted into the space between it and the supporting tube, and when the wax sets the porometer chamber is firmly held and the leaf chamber is air-tight.

To measure the rate at which air could be drawn through the leaf the *air-flow meter* shown in fig. 1, B, was used. Air suction is produced by means of a column of mercury, about 15 mm. long, falling vertically in the straight limb of the flow meter, and the time taken for the mercury to pass a marked distance down the tube is observed with a stop-watch. One limb of this meter is attached by pressure tubing to the porometer chamber, the other to a "T" piece, which puts it into communication with the leaf chamber. The whole apparatus therefore forms a closed system. After making a reading the mercury is returned, by inverting the tube, to its proper end, and the tube is then set horizontal until the next reading. Readings were always taken in the direction in which air is *drawn* through the leaf.

A great advantage of the gelatine-disc method of securing contact between the porometer chamber and the leaf is that, before setting up the assimilation experiment, readings can be rapidly taken with the same disc over the whole leaf surface. Usually three readings on each side of the main vein were thus taken. Unless all six readings were fairly close to one another, the leaf was not used.

Similar readings at the end of the experiment enabled one to check the reading for the part of the leaf used during the experiment against the other parts of the leaf. Usually the average reading for the rest of the leaf agreed well (after the experiment as well as before it) with that for the area used in the experiment. There were cases, however, of marked divergence; the stomata of the rest of the leaf might be either more widely open or more closely shut than those of the area within the porometer chamber. Several experiments had to be rejected on these grounds.

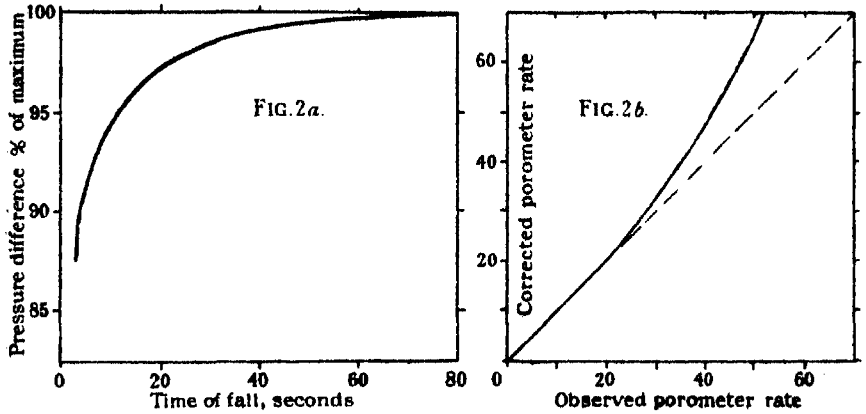
In all the experiments to be described, unless a special note is made to the contrary, it may be taken that the average porometer rate for the leaf surface both before and after the assimilation experiment did not differ by more than 10 per cent. from the rate given by the area that was under the porometer chamber during the experiment.

In calculating the assimilation per 50 sq. cm. of leaf per hour the area of leaf surface covered by the gelatine disc (about 4 sq. cm.) is subtracted from the area of the leaf. During an assimilation experiment porometer observations were usually made at half-hour intervals. Two readings were taken at each time of observation, and the mean of these recorded. The volume of air drawn through the leaf in making a single reading is less than 1 c.c., so that in a period of an hour, during which 1500 c.c. of air, enriched with CO_2, pass through the assimilation chamber, less than 4 c.c. of the air in the assimilation chamber are drawn through the leaf into the porometer chamber. Any effect, therefore, of the short periods of air flow upon uptake of CO_2 by the leaf is quite negligible.

(*b*) *Calculation of Porometer Rates.*

The "*observed porometer rate*" is defined as $\sqrt{(10^4/\mathrm{T})}$, T being the time in seconds for the mercury column to fall through 2 inches* in the vertical limb of the flow meter, for a porometer area† of 0·65 sq. cm. For use as a measure of changes in stomatal opening, certain corrections are necessary.

In the first place, at rapid rates of fall, the full pressure of the mercury column is not maintained, owing to the existence of a resistance between the mercury and the wall of the glass tube. An empirical relation between rate of fall and the pressure maintained was established by the use of a paraffin manometer to measure the pressure difference maintained by the falling mercury (fig. 2, A).



FIGS. 2A and 2B.

FIG. 2A.—Relation between the pressure difference maintained by the falling mercury column, in the flow meter, and the time taken by the column to fall 2 inches.

FIG. 2B.—Correction of observed porometer rates for the variation of pressure difference with time of fall, and for intercellular space resistance. The magnitude of the correction may be judged by the divergence between the continuous line (corrected values) and the broken line (observed values).

By varying the pressure difference employed, the flow of air, within the observed range of stomatal opening and at pressure differences of the order employed experimentally, was shown to have the characteristics of "viscous flow"—the rate of flow being proportional to the pressure difference employed.

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* For very slow rates of fall the time of fall was measured over a shorter distance (usually 0·4 inch) and multiplied by the appropriate factor.

† The porometer area is the area of leaf surface exposed *within* the porometer chamber.

Correction can therefore be quite simply made for the variation in pressure-difference with variation in rate of fall.

The second factor which needs consideration is the resistance offered to the flow of air by the intercellular spaces of the leaf. The observed time for the flow of a given volume of air will be proportional to the sum of the stomatal and the intercellular space resistances. Experiments with a double porometer chamber* on the Cherry Laurel leaf showed that, if the minimum horizontal distance between entry and exit of air is increased by 9 mm., then the time for flow of the standard volume of air in the flow meter is increased on the average by 1·4 seconds (the values at widely differing rates of flow fluctuating fairly closely about this mean value). A correction based upon this result has therefore been made for the experimental porometer rates; the corrected values are accordingly believed to be a close approximation to the square root of the capacity for air-flow of the stomata on 0·65 sq. cm. of leaf surface. These corrections only affect to any appreciable extent the more rapid rates of flow; the magnitude of the correction is shown by fig. 2B (p. 491).

Further, the flow meter was standardised against a capillary tube of known dimensions, so that the observed rates of flow can be stated in absolute units. Calculation of the coefficient of viscosity of air from the dimensions of the capillary tube, the rate of flow of air, and the pressure difference employed, gave for this coefficient the value $18{\cdot}4 \times 10^5$—in close agreement with the established value. Use will be made of these absolute units at a later stage.

While the experiments to be described in this section were in progress, a rough attempt was made to establish the fact that the observed variations in porometer rates were, *in fact,* due to variations in stomatal opening and, if possible, to establish the type of relation existing between porometer rate and stomatal opening. Lloyd's "strip" method was not found to give clear results for the stomatal openings, and more useful results were obtained by means of wax impressions. Porometer readings were taken on a leaf, the porometer chamber and gelatine disc were removed, and, on the area previously exposed within the porometer chamber, wax impressions were at once taken. The dimensions of the stomatal pores were measured by means of a dry ⅛-inch objective and a micrometer eyepiece. Successful impressions were rare, and only three satisfactory results were obtained.

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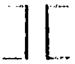
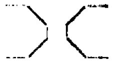
* See Knight (10).

These are shown in the table below :—

Porometer rate = $\sqrt{\frac{10^4}{T}}$	Product of major and minor semi-axes of pore. (Average of 30.)
22·36	11·3 μ^2
18·90	9·64 μ^2
9·54	5·48 μ^2

The results were taken to confirm the belief that variations in porometer rate are occasioned by variations in stomatal opening, but were regarded as insufficient for any conclusions with regard to the form of the relation between porometer rate and pore area.

The really important question is not so much the relation between porometer rate and area of stomatal pore as that between porometer rate and *diffusive capacity of stoma*. This question was subsequently explored both experimentally and theoretically in collaboration with Mr. G. E. Briggs for the purpose of joint work on the apparent assimilation of whole plants* and cannot be considered in detail here. The theoretical problem is a complex one : for a pore of circular cross-section, and dimensions of the order of those found in Cherry Laurel and Vicia, the square root of the capacity for viscous flow of air appears to give the simplest reasonable approximation to the diffusive capacity of the stoma, whether the sides of the stoma in vertical section be straight, thus.  or sloping, thus . Most stomata, however, appear to become narrowly elliptical as they close, and this change of cross-sectional form disturbs the simple relationship. There are, however, certain compensating factors which tend to minimise this distortion of the simpler relationship. One of these, namely, variability in opening within a group of stomata on the leaf surface, will be referred to in a later section. For the present the square root of the stomatal capacity for flow of air is taken as representing the closest simple approximation that can be made to an experimental measure of stomatal diffusive capacity.

It may be noted here that, even if it were possible to make frequent measurements of average stomatal pore area on a leaf, during a transpiration or assimilation experiment, there would still remain the complex problem of the calculation of stomatal diffusive capacity, involving a knowledge of the depth of the pore, the shape in vertical and cross-section, and the way in which the shape changes with opening.

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* Unpublished work by G. E. Briggs and E. J. Maskell. The writer wishes to thank Mr. Briggs for his ready agreement to the inclusion here of the above brief reference to the results of that work.

One further point must be made before proceeding to a description of the experimental results. The rate of flow of air through 0·65 cm. of leaf surface will be a function, not only of the average stomatal opening, but also of the number of stomata. Consequently if, from the porometer rate, an estimate is to be made of variations in *stomatal diffusive capacity per unit surface area* of leaf, correction must be made for any variations between different leaves in number of stomata per square centimetre. Measurements were therefore made of the number of stomata per square centimetre for most of the experimental leaves, both for the porometer area and for the rest of the leaf. For the purpose of graphs involving more than one leaf and showing the observed relation between stomatal opening (as measured by porometer rate) and rates of apparent assimilation (per 50 sq. cm. per hour), the porometer rates have been so adjusted as to take this factor into account. The "*adjusted porometer rates*" are so calculated as to be proportional to the stomatal diffusive capacity of unit area of leaf surface.*

Calculation is made as follows, all rates being adjusted to the standard of Experiment LI (*v.* p. 495):—

Adjusted porometer rate = observed porometer rate multiplied by

$$\frac{\mathrm{N}}{\sqrt{(\mathrm{N}p)}} \times \frac{\sqrt{(\mathrm{N}p_{\mathrm{LI}})}}{\mathrm{N}_{\mathrm{LI}}},$$

where N = number of stomata per square centimetre of leaf surface on area exposed outside porometer chamber; $\mathrm{N}p$ = the same value for the surface inside the porometer chamber; and N_{LI} and $\mathrm{N}p_{\mathrm{LI}}$ are the corresponding values for Experiment LI.

(*c*) *The Relation between Porometer Rate and Rate of Assimilation, December*, 1920.

The results of the first two experiments with the porometer apparatus described in the previous section are shown in figs. 3 and 4. As in all the subsequent experiments on the relation between porometer rate and assimilation (except where the effect of change in light intensity is under investigation) the "high" light intensity was employed; and the concentration of CO_2 supplied was

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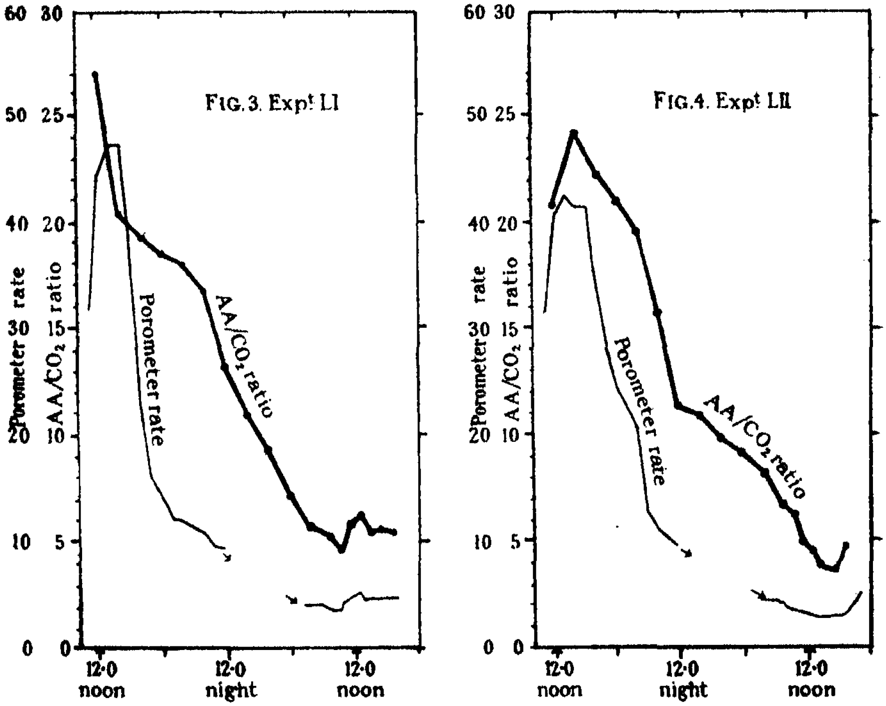
* Assuming, of course, the validity of the equation for each stoma—Diffusive capacity = $\sqrt{}$(capacity for air flow).



Figs. 3 and 4.—March of assimilation and of porometer rate, December, 1920. In this *as in all subsequent graphs of the diurnal march,* A.A./CO$_2$ ratios are shown by thick lines, porometer rates by thin lines, and the vertical scale for porometer rate is *one-half* that for the A.A./CO$_2$ ratio.

Fig. 3.—Experiment LI, B.G., December 6–7. "High" light: average CO$_2$ = 0·230 per cent.; temperature 19·5–21·2° C.

Fig. 4.—Experiment LII, B.G., December 8–9. "High" light: average CO$_2$ = 0·245 per cent.; temperature 19·2–20·4° C.

such that the maximum values of assimilation should be well below the light limited value. Following the procedure of the previous paper (12) the march of assimilation under these conditions is expressed in terms of the A.A./CO$_2$ ratios (ratio of apparent assimilation to mean CO$_2$ supply) for each assimilation reading. The thin lines, in figs. 3 and 4, show the porometer rates—the heavy lines show the A.A./CO$_2$ ratios. The two curves show a fair agreement; a slight rise on the second morning is evident in both curves, and is almost the last sign which the 1920 leaves showed of the rhythm of recovery from the nocturnal depression which characterised them from July to November.

In the early part of the experiments the stomatal curve falls more rapidly than the assimilation curve. This relation is shown more clearly in fig. 5, where the A.A./CO_2 ratios have been plotted against the mean values of the porometer rate for the corresponding periods. Up to a porometer rate of about 10 the relation appears to be approximately linear. Above this the assimilation values rapidly fall away, and seem to approach asymptotically to a maximum. Taking into consideration the possibility that stomatal changes in the porometer area may not always run exactly parallel with those on the rest of the leaf, the values appear to fall pretty well on to a curve of quite definite form, and, moreover, the two experiments show a large measure of agreement. (This agreement between experiments of about the same date was noted in the previous paper for the diurnal march of assimilation in these leaves, and it extends also to the form of the assimilation rate-porometer rate curves.)

The general form of the observed relation between stomatal opening (as measured by porometer rate) and apparent assimilation is a consequence of the fact that the stomata form only a part of the total resistance to the uptake of CO_2 by the leaf. The remaining resistances include the resistance to diffusion in the air path up to the leaf surface, the resistance of the intercellular space system, of the cell walls and protoplasm up to the chloroplast surface, and, in addition, the "resistances" in the photochemical and chemical phases of the process of photosynthesis. The rate of uptake of CO_2 by unit area of leaf, for unit external concentration of CO_2, will be proportional to the reciprocal of the sum of these resistances per unit area.

For the purpose of the present discussion it will be sufficient to write A.A./CO_2 ratio $= \frac{k}{S + L}$, where S is the stomatal resistance per unit area, and L is the sum of the remaining resistances of the leaf per unit area. When S is great compared with L, a small change in S will produce an almost proportionate change in the whole expression. As the stomata open and S becomes small, changes in S have less and less effect, and the values of the A.A./CO_2 ratio tend to the limit, k/L. The exact calculation of the stomatal resistance is one of extreme difficulty—the reciprocal of the porometer rate appears, however, the nearest experimental approximation to this value which can be made, and the writer has ventured an approximate solution of the experimental results in those terms. The thirty values of Experiments LI and LII have been used to solve, by a least-square method, an equation of the form A.A./CO_2 ratio $= \frac{1}{k_1 + k_2/P}$, in which k_1 corresponds to L, and k_2/P to S in the previous equation, P being the porometer rate. The equation calculated is A.A./CO$_2$ ratio $= \dfrac{1}{0{\cdot}0265 + 0{\cdot}591/\mathrm{P}}$, and the calculated curve is shown in fig. 5 as the continuous line.

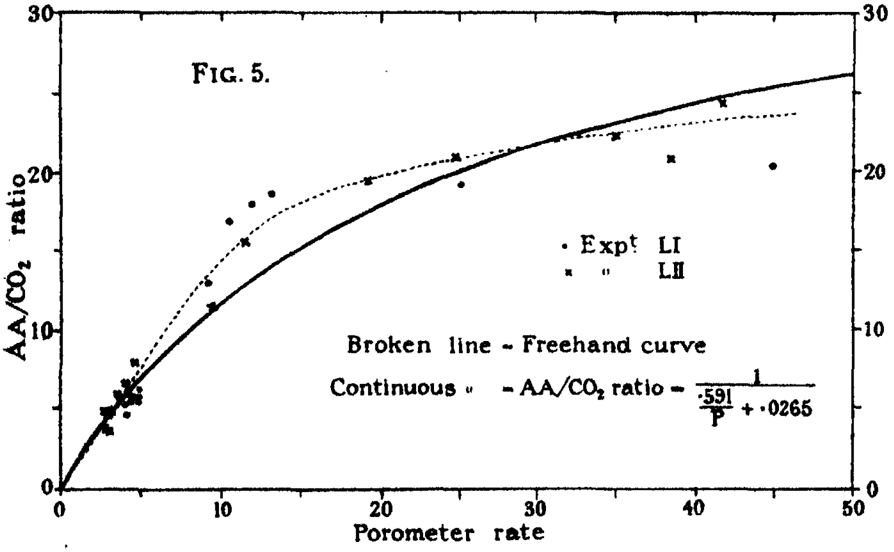


Fig. 5.—Relation between porometer rate and apparent assimilation at "high" light, December, 1920. (As in all subsequent graphs of this relation, *the scales for porometer rate and* A.A./CO$_2$ *ratio are of equal magnitude.*) Experiments LI and LII (see figs. 3 and 4).

No more is claimed than that the equation provides a first approximation to an analysis of the data, and that it has a reasonable theoretical basis. Three assumptions are involved in its use. The first, which is inevitable if any use is to be made of the data, is that changes in stomatal opening in the porometer area approximately represent changes in the stomatal opening of the rest of the leaf. The precautions taken in technique already mentioned (p. 490) and subsequent experiments give reasonable grounds for this assumption. The second assumption is, that the remaining resistances of the leaf are approximately constant under the experimental conditions (light constant, temperature and rate of air current approximately constant). As regards the resistances in the diffusion phase, the assumption is not unreasonable, and in so far as the "resistances" in the photochemical and chemical phases of the process are a function of light and temperature, the assumption is justified for these also. But these "resistances" may also be a function of the CO$_2$ concentration at the chloroplast surface, especially as the rate of apparent assimilation approaches the temperature or light-limited values. A more complete formula for the equation would then be A.A./CO_2 ratio $= \dfrac{1}{k_1 + k_2 \dfrac{1}{P} + k_3 f(P)}$; since, with constant external concentration of CO_2, the concentration at the chloroplast surface will increase with increase in P. An equation of this form would give a curve, deviating from the curve for the simpler form of equation, somewhat in the same way as does the freehand curve drawn through the experimental points (see fig. 5). As the relation between rate of assimilation and CO_2 concentration just outside the chloroplast surface is still, however, an unsettled problem,* the exact solution of this question must be deferred (see later, p. 527).

The third assumption is that the porometer rate is inversely proportional to the stomatal resistance to diffusion. While this appears to be the nearest empirical approximation to the correct relation, theoretical considerations suggest that where eccentricity of the pore opening is important, the stomatal capacity for diffusion will increase less rapidly than the porometer rate. In the absence of precise knowledge on these two points, the simpler form of equation is retained as a provisional method of analysing the data.

(*d*) *Further Experiments on the Relation between Porometer Rate and Rate of Assimilation.*

Not all the experiments show the relatively simple relation between porometer rate and rate of assimilation shown in fig. 5. Experiment LXXVIII, May 5–6, approaches it closely (figs. 7 and 8), although three of the values in the middle region of stomatal opening (see fig. 8) lie well below a straight line joining the higher values to the origin. The stomata are, however, shutting very rapidly at the end of the experiment, and close agreement would not be expected. In Experiment LXXIV, April 26–27, the divergence is of a slightly different type (figs. 6 and 8). The values for the first day fall pretty well on to a curve of the usual form, but all the assimilation values on the morning of the second day are too low. The freehand curve drawn for this experiment neglects these values, but a curve drawn through the experimental points would apparently cut the horizontal axis at some point to the right of the origin. Clearly, during the experiment, parallel with stomatal closure, there is proceeding an increase in the other resistances of the leaf.

Using the terms of the equation above A.A./CO_2 ratio $= \dfrac{k}{L + S}$, in this

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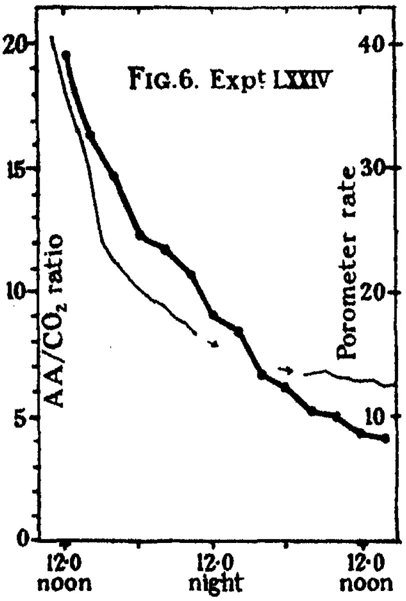
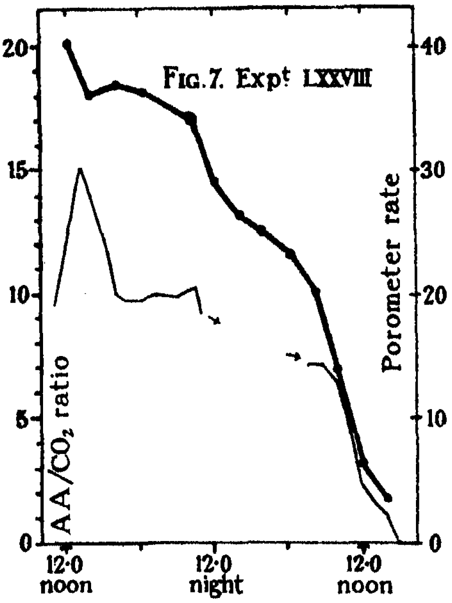
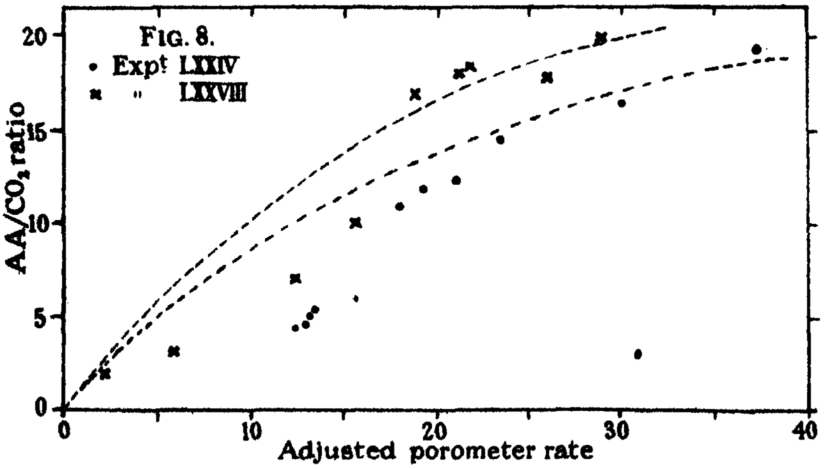
* In the experiments of Harder, 1921 (7), and of Warburg, 1919 (15), only a theoretical external concentration of CO_2 is known, and there may be an appreciable diffusion path to be taken into account (*v.* pp. 521–522).







Fɪɢs. 6, 7 and 8.—March of porometer rate and of apparent assimilation, April and May 1921.

Fɪɢ. 6.—Experiment LXXIV, B.G., April 26–27, 1921. "High" light: average CO_2 = 0·353 per cent.; temperature 21–23° C.

Fɪɢ. 7.—Experiment LXXVIII, B.G., May 5–6, 1921. "High" light: average CO_2 = 0·129 per cent.; temperature 19·8–22·6° C.

Fɪɢ. 8.—Relation between porometer rate and assimilation for Experiments LXXIV and LXXVIII (figs. 6 and 7).

experiment L is increasing with time, at first somewhat less rapidly than S, later more rapidly than S. It does not appear probable, from the evidence discussed in the earlier paper* and from similar experiments in 1921, that this increased resistance is in general to be attributed to changes in the photochemical or chemical phases of photosynthesis. What appears more probable is a change in the resistance to diffusion offered by the cell wall and protoplasm. It is a possibility to be borne in mind that such changes may in some cases run parallel with changes in stomatal resistance—especially in the case of so marked a variation in stomatal resistance as is shown by the rhythm of nocturnal closure.

Curves of each of the three types shown in figs. 3–8 were obtained at different seasons: in early January, 1921, curves like those of figs. 3, 4 and 5 for December, 1920; in late January and April, curves like those of April 26–27 (figs. 6 and 8); in May, curves like those of May 5–6 (figs. 7 and 8). The close agreement between the results of experiments of about the same date is again a noteworthy feature of this material.

(e) *The March of Assimilation Rates and Porometer Rates in Leaves appearing in* 1921—*Effect of Drought.*

A more complete series of curves can be shown for leaves which appeared in May, 1921. Experiment XCIV, fig. 9, June 17–18, 1921: the A.A./CO_2 ratios show high initial values, a rapid fall to 12 midnight, and only a very slight morning recovery. The stomatal curve follows the assimilation curve quite closely.

The summer of 1921 was characterised by its very low rainfall, and experiments during July and August showed very low porometer rates associated with very low assimilation rates, and the absence of that marked rhythm of nocturnal depression and morning recovery which marked the experiments in the same months of 1920. This condition persisted until October 4–5, when the break of the drought began.

Fig. 10 (Experiment CI, October 4–5) shows the form of curve typical of the summer period up to the break of the drought. On the evening after these leaves had been picked nearly half an inch of rain fell. Fig. 11 (Experiment CIII, October 7–8) (this leaf having been picked two days after the rain) shows again the high initial values, both of assimilation and of porometer rates, noted in the June experiment and characteristic of the previous year's results for this season. No recovery, however, either in assimilation or in porometer

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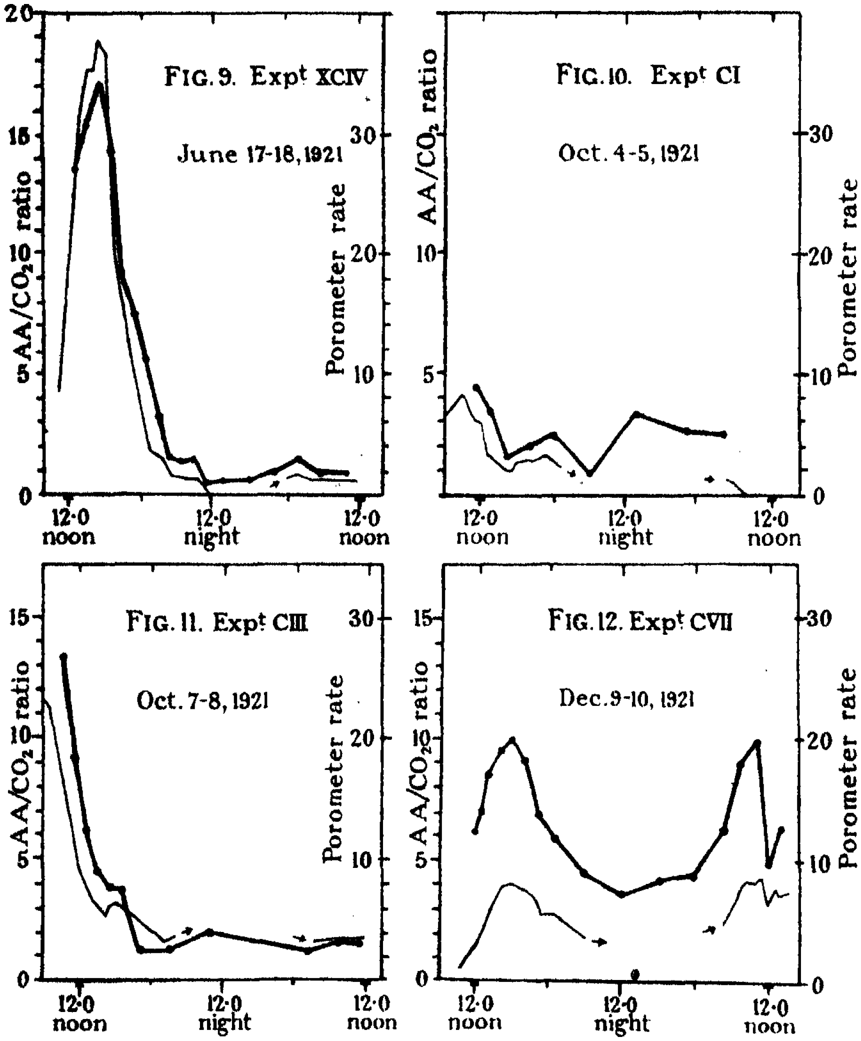
* *Loc. cit.*, pp. 474, &c.



FIGS. 9, 10, 11 and 12.—March of assimilation and porometer rate for leaves appearing in 1921.

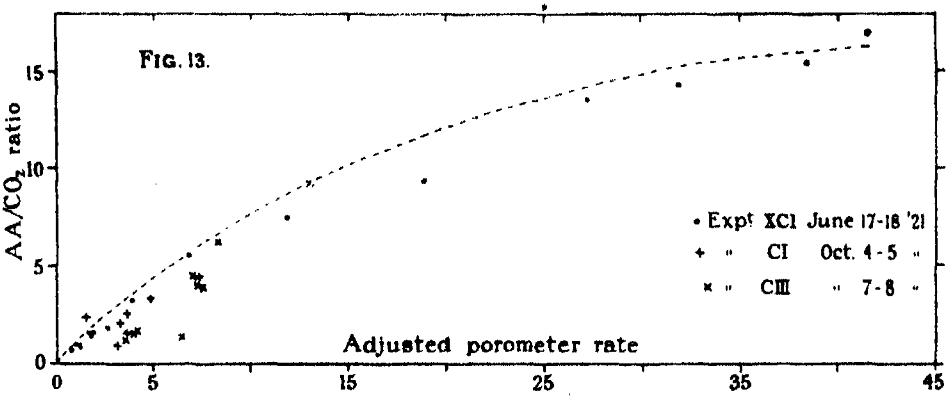
FIG. 9.—Experiment XCIV, B.G., June 17–18, 1921. "High" light: average CO_2 = 0·290 per cent.; temperature 21·4–24° C.

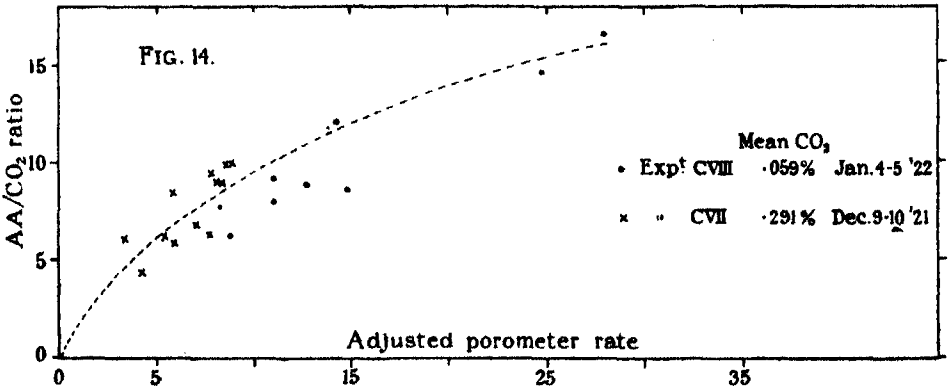
FIG. 10.—Experiment CI, B.G., October 4–5, 1921. "High" light: average CO_2 = 0·330 per cent.; temperature 21·9–24° C.

FIG. 11.—Experiment CIII, B.G., October 7–8, 1921. "High" light: average CO_2 = 0·293 per cent.; temperature 23·4–24·6° C.

FIG. 12.—Experiment CVII, B.G., December 9–10, 1921. "High" light: average CO_2 = 0·291 per cent.; temperature 19·8–20·6° C.

rate, occurs on the second morning. The relation between porometer rate and assimilation for this set of experiments is shown in fig. 13. Experiments CI and CIII, during the drought, agree roughly with Experiment XCIV, before the drought, in the position of their initial values on the assimilation-porometer rate curve, but the later assimilation values of both these experiments fall definitely too low. Again, an increased resistance on the part of the mesophyll cells of the leaf to the uptake of CO_2 is indicated, and it seems reasonable to regard it as an effect of the drought.





FIGS. 13 and 14.—Relation between porometer rate and assimilation, summer, 1921, and winter, 1921–1922.

FIG. 13.—June and October, 1921. Experiments XCIV, CI and CIII (see figs. 9, 10 and 11).

FIG. 14.—December, 1921, and January, 1922.

Experiment CVII (see fig. 12).

Experiment CVIII, B.G., January 4–5, 1922. "High" light: average $CO_2 = 0 \cdot 059$ per cent.; temperature 18–19·4° C.

For the appearance of the typical rhythm shown in 1920, we have to wait until December. Fig. 12 (Experiment CVII, December 9–10) shows the first appearance of the full rhythm with morning recovery in the leaves of 1921. An experiment done shortly afterwards—January 4–5, 1922 (Experiment CVIII)—at an average CO_2 of 0·059 per cent. only, was of interest in showing that the existence of the rhythm and the general magnitude of the values of the A.A./CO_2 ratios observed in this set of experiments were not peculiar to the particular range of CO_2 concentrations employed. Fig. 14 shows the adjusted porometer rate for these two experiments plotted against the A.A./CO_2 ratio. The data of two experiments appear essentially to form parts of the same curve relating porometer rate to assimilation, though the January experiment shows an advance on the December one in range of stomatal opening and of assimilation rates, just as the December one showed an advance on those during the period of drought July to October.

(*f*) *The Daily March of Assimilation and of Porometer Rates, July*, 1922.

Having brought the series back to the re-establishment of the rhythm with which, in a sense, the problem started, no further experiments of this straightforward type were attempted until July, 1922, when, on leaves appearing in that year and developing under more favourable moisture conditions, it was hoped to obtain final evidence for the stomatal explanation of the rhythm shown so markedly in the summer of 1920. Fig. 15 (p. 504) shows the result of this experiment (Experiment CXI), which lasted from July 12, 7.30 a.m., to 4 p.m. on July 15. It was impossible to make an unbroken series of estimations of the assimilation or of porometer rates, owing to the pressure of other work, but the evidence is sufficiently conclusive. In both curves there is a preliminary rise, followed by a rapid fall until 3 p.m., and that succeeded by a slight temporary rise. After 8 p.m. the experiment was left running, but no estimations were made until the next morning. By 8 a.m. on the 13th the porometer rate had recovered from its low night value and showed a rapid rise to a maximum, at about 11.30 a.m.

Before recommencing the assimilation readings the leaf was removed from the chamber, weighed, and porometer readings taken over its surface. These, as on the subsequent occasions during the experiment on which this was done, agreed remarkably well. At 11 a.m. assimilation readings recommenced. The high initial value is what would be expected from the porometer rate, and the two curves show similar behaviour up to 10 p.m., when the porometer readings stop. It will be noted that the nocturnal depression is not so great, though it is quite as well marked, as on the first night. Porometer readings begin again at 7 a.m. July 14, and show the usual recovery from the night values. Thereafter, however, with some fluctuations, they remain high, and the characteristic rhythm is absent. At 6 p.m. they are still very high.

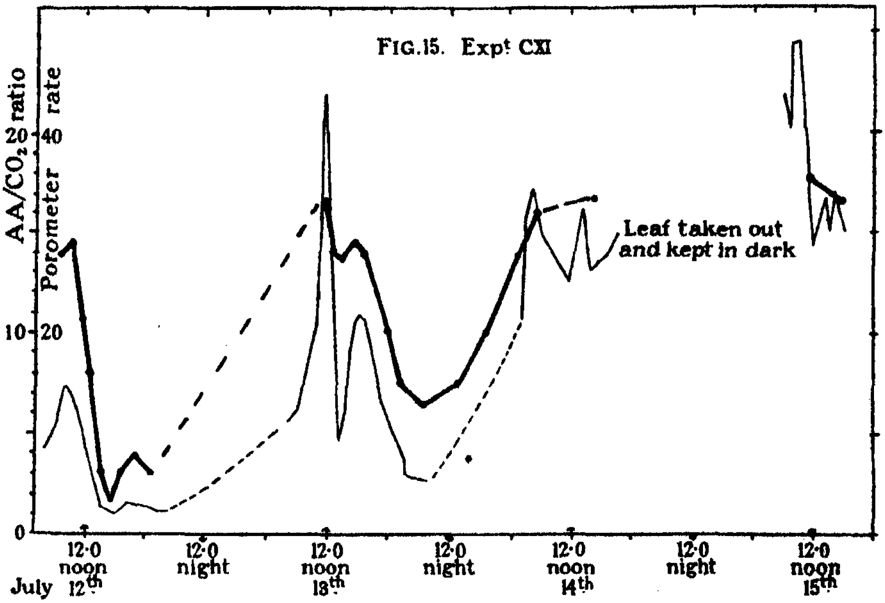


FIG. 15.—March of assimilation and of porometer rate, summer, 1922. Experiment CXI, B.G., July 12–15, 1922. "High" light: average $CO_2 = 0 \cdot 285$ per cent.; temperature 22–23·9° C. Periods during which no readings were taken are indicated by the broken lines on the curves.

During this day some readings of apparent assimilation at high CO_2 concentrations were taken; these are omitted from the curve, values for $A.A./CO_2$ ratios being given only for periods of low CO_2 concentration, with assimilation rates well below the light-limited values. At 6 p.m. on the 14th the leaf was removed from the assimilation chamber and kept in a moist tin until next morning. On this day two assimilation readings at "high CO_2," alternating with two at "low CO_2," were made. The two readings at low CO_2 give values of the $A.A./CO_2$ ratio which fall into line with the results for the first day. It will be seen that the porometer readings remain high on this day.

Fig. 16 shows the relation between porometer rate and assimilation for this experiment, and the calculated curve of nearest fit of the form

$$A.A./CO_2 \text{ ratio} = \frac{1}{k_1 + \frac{k_2}{P}}.$$

The fit appears to be closer than in the case of Experiments LI and LII (p. 495), but again there is the same kind of divergence between the apparent relation and the calculated curve. A freehand curve would rise rather more steeply and "flatten out" somewhat more abruptly. In general, this experiment repeats for the first two days the rhythm of assimilation shown in the summer of 1920, and shows that it is paralleled by a rhythm of stomatal behaviour. The form of the assimilation-porometer rate curve repeats that of December, 1920, and, like that, is not complicated by that increase with time in non-stomatal resistance to diffusion, shown by leaves during the drought of 1921.

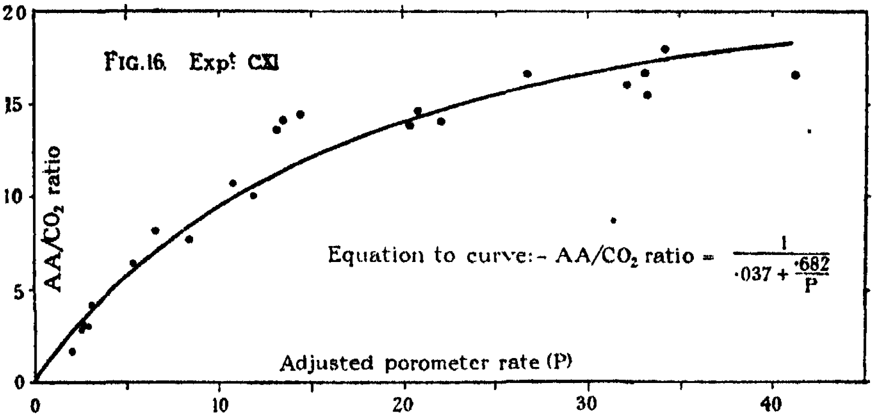


FIG. 16.—Relation between assimilation and porometer rate, July, 1922. Experiment CXI (see fig. 15).

(*g*) *Experiments with the Porometer on Leaves taken direct from the Bush.*

In the summer of 1920 leaves taken direct from the bush at 9 a.m. or 10 a.m., and set up at once in the assimilation chamber, showed a marked depression in assimilation—a "first minimum"—at about 2 p.m. or 3 p.m.* Only after recovery from this first fall did the characteristic nocturnal depression begin. It was of interest to see whether this "first minimum" was, like the nocturnal fall, to be attributed to stomatal behaviour. Accordingly, in 1921, after the break of the drought, experiments were done on "direct leaves," using the porometer. The results of two such experiments are shown in figs. 17 and 18 (p. 506).

Experiment CII was on a Botany School leaf (fig. 17A) and Experiment CIV on a Botanic Garden leaf (fig. 17B). Hourly readings of assimilation were made up to 9 p.m. or 10 p.m.; after that longer intervals were used. There was, as in the case of the "24 hour leaves" at this season in 1921, no recovery in

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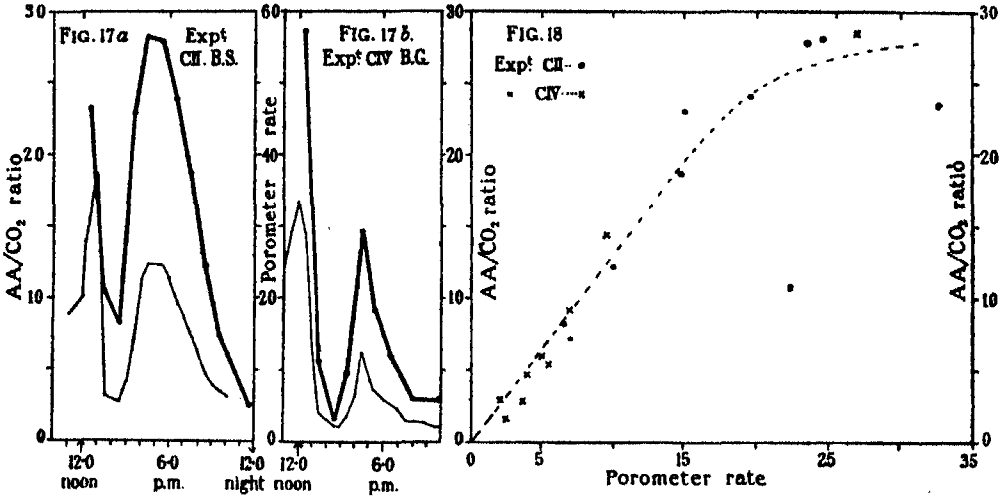
* See earlier paper (12).



FIGS. 17A, 17B and 18.—March of assimilation and of porometer rate for leaves set up direct from the bush.

FIG. 17A.—Experiment CII, B.S., October 5–6, 1921. "High" light: average $CO_2 = 0{\cdot}246$ per cent.; temperature 23·3–24·6° C.

FIG. 17B.—Experiment CIV, B.G., October 11–12, 1921. "High" light: average $CO_2 = 0{\cdot}317$ per cent.; temperature 24·2–25° C.

FIG. 18.—Relation between assimilation and porometer rate for "direct leaves." Experiments CII and CIV (see figs. 17A and 17B).

the morning, and the results are only plotted up to midnight. In spite of the very rapid changes, both of assimilation rate and of porometer rate, the parallelism between the march of assimilation and the march of stomatal opening is remarkably close, as may be seen also from fig. 18, which shows the relation between porometer rate and assimilation rate for these two experiments. It is clear that the "first minimum" is a stomatal phenomenon.

It may be noted that the maximal values of the A.A./CO_2 ratio lie very high, especially in comparison with the "24-hour leaves"* of the same season (*cf.* fig. 13). This indicates some difference between "direct leaves" and the "24-hour leaves" in the non-stomatal resistances of the leaf. Time was not available for further exploration of this point; the experiments were designed merely as a further test of the stomatal explanation of the major variations observed during the march of assimilation of a leaf, at "limiting" concentrations of CO_2.

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* Leaves kept 24 hours before setting up in the leaf chamber (see previous paper).

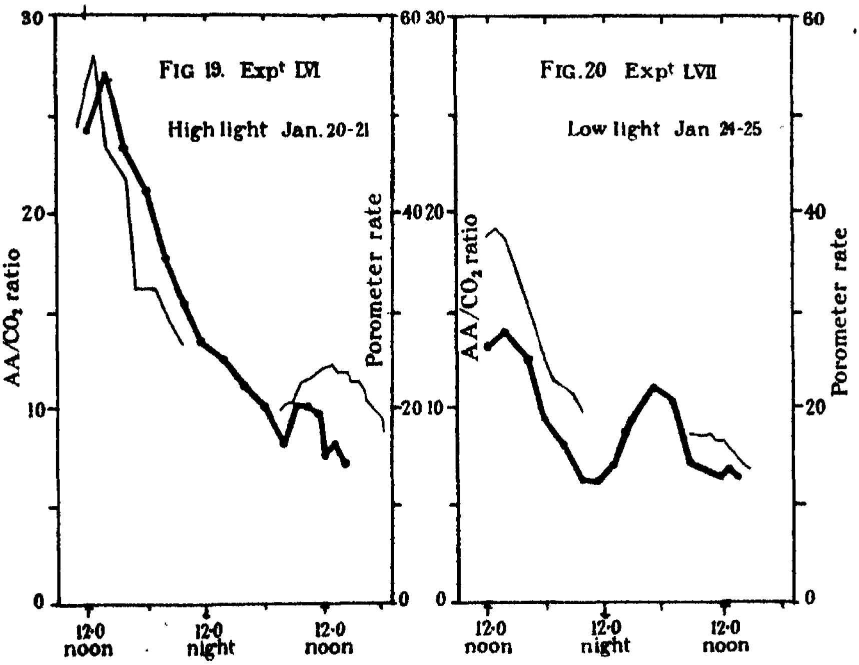
(*h*) *The Effect of Light Intensity upon Assimilation Rate and upon Porometer Rate.*

Among those characteristics of the assimilation curves, which suggested the tentative conclusion advanced in the earlier paper, that the major variations in the A.A./CO_2 ratios were due to changes in stomatal opening, was their relation to light intensity. It was shown

(1) That comparing experiments done at proximate dates, the diurnal curve for the "high" light intensity ran definitely above that for the "low" light intensity; and

(2) That by increasing or decreasing the light intensity during the course of an experiment, the A.A./CO_2 ratios could similarly be increased or decreased.

Experiments similar to those described in the earlier paper (*loc. cit.*, p. 481) but now using the porometer, were done to test whether the whole of this effect was, in fact, due to the influence of light intensity upon stomatal opening.



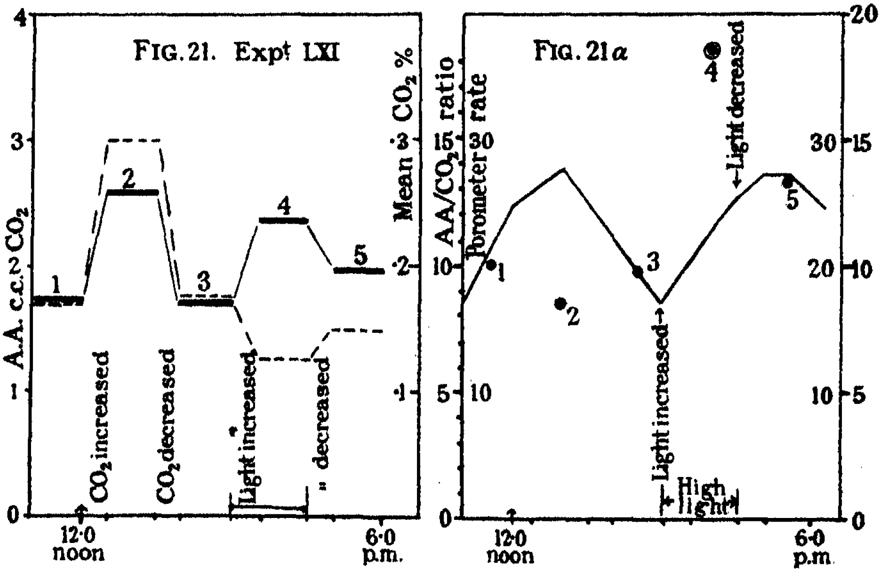
FIGS. 19 and 20.—March of assimilation and porometer rate at "high" and at "low" light intensities.

FIG. 19.—Experiment LVI, B.G., January 20–21, 1921. "High" light: average CO_2 = 0·192 per cent.; temperature 19·6–21° C.

FIG. 20.—Experiment LVII, B.G., January 24–25, 1921. "Low" light: average CO_2 = 0·142 per cent.; temperature 20–21·8° C.

Two experiments (LVI, January 20–21, fig. 19, and LVII, January 24–25, fig. 20) at " high " light and " low " light respectively, are sufficiently close in time—within four days of one another—to be compared. Both show the phenomenon noted earlier in this paper for some April experiments (see p. 498), of assimilation values on the second morning which fall below those to be expected from the porometer rates. It is quite clear, however, that both assimilation rates and porometer rates are higher at the " high " light intensity, and also that at " low " light intensity the assimilation rates are depressed to a greater extent than are the porometer rates.

It was important to confirm this result, and two further experiments were done, during February, in which changes of light intensity were made during the course of an experiment. The results may be illustrated by means of fig. 21 (Experiment LXI, February 18), which shows the values of the apparent assimilation and of mean CO_2 per cent. for *one* of these experiments. Hourly



FIGS. 21 and 21A.—The effect of change in light intensity upon porometer rate and upon assimilation.

FIG. 21.—Experiment LXI, B.G., February 18, 1921. Light " low " from 11 a.m. to 3 p.m.; " high " 3 p.m. to 4.30 p.m.; then " low." Mean CO_2 per cent. is shown on the graph by the dotted line, apparent assimilation by the thick continuous lines. The readings of assimilation are numbered in serial order.

FIG. 21A.—A.A./CO_2 ratios and porometer rates for Experiment LXI. The A.A./CO_2 ratios are shown by dots numbered to correspond with the assimilation periods (see fig. 21).

readings of assimilations were made, and when conditions were changed, an interval of half an hour was allowed before commencing the next reading.

In these experiments it was first shown that increased CO_2 concentration (the second reading) increases the apparent assimilation (in both experiments to the same value—the light-limited value). The original CO_2 concentration is re-established; the assimilation falls. Proof is thus given that, at the lower CO_2 concentration, CO_2 is "limiting" the rate of assimilation. The light intensity is now increased; the apparent assimilation increases. The light is decreased; the apparent assimilation also decreases. The porometer rates and the A.A./CO_2 ratios for Experiment LXI are given in fig. 21A. The A.A./CO_2 ratios are numbered to correspond with the five assimilation periods shown in fig. 21 and the value for "high" light (No. 4) is distinguished by a circle. The second value is for the period at high CO_2 concentration (CO_2 not "limiting") and cannot be used for comparison with the others. It will be seen that increased light intensity is indeed accompanied by increased stomatal opening, but that the effect upon the A.A./CO_2 ratio is still greater. The second experiment of the same type (Experiment LXII, February 22) gave exactly similar results.

The results for these four experiments—LVI, LVII, LXI, LXII—are summed up in fig. 22, in which the A.A./CO_2 ratios (for those values of the assimilation which are "CO_2 limited") are plotted against the "adjusted" porometer

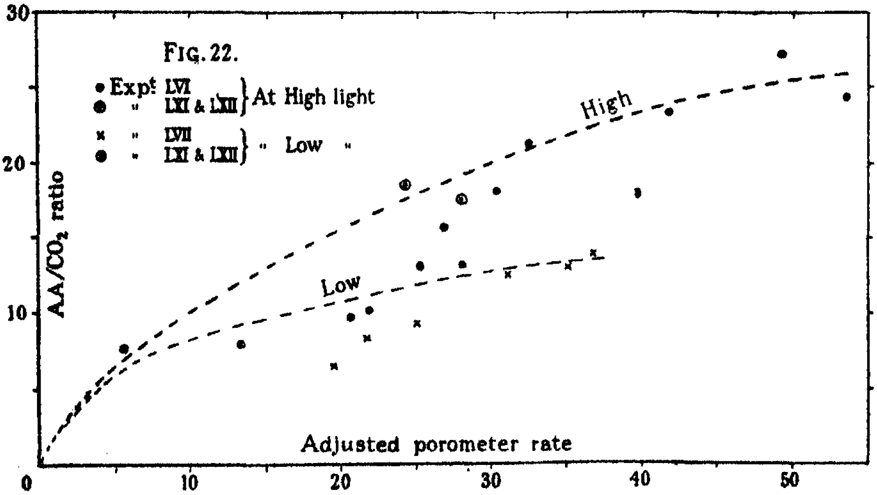


FIG. 22.—Relation between assimilation and porometer rate at "high" and at "low" light intensities. This graph groups the results for Experiments LVI, LVII, LXI (see figs. 31, 32 and 33). Also for Experiment LXII, B.G., February 22, 1921, which is of similar type to LXI.

rates. (Only the values of the first day for Experiments LVI and LVII are plotted.)* It is clear that we are dealing with two distinct curves, which approximate to one another at low porometer rates, but tend towards quite distinct maxima, which are determined by the light intensity, though they are by no means proportional to it.

Reverting to the expression A.A./CO_2 ratio $= \frac{k}{S + L}$ (p. 496), we may say that light has its effect upon the apparent assimilation, when CO_2 is "limiting," by diminishing both S, the stomatal resistance to diffusion, and L, the remaining resistances of the leaf. Whether this effect upon the non-stomatal part of the resistances in the process of assimilation be one upon the resistances in the liquid diffusion phase (from intercellular space through cell wall and protoplasm to chloroplast surface), or upon those in the photochemical or chemical phases of the process, cannot, in the present state of our knowledge of the dynamics of photosynthetics, be exactly decided, since neither in the work of Warburg nor in that of Harder is the problem introduced by the resistance in the liquid diffusion phase satisfactorily dealt with.

Some attempt will be made to discuss the work of these workers and of Lundegårdh in a later section, but it may be said here that *an* effect of light upon each of the resistances mentioned appears to the present writer highly probable. The effect upon the "resistances" in the photochemical phase may be taken for granted, although the exact expression of this effect in quantitative form is by no means easy. In addition, the temperature of the assimilating cell must inevitably increase with increased illumination, and some reduction in the "resistances" in the chemical phase and probably also in the liquid diffusion phase must result. Finally, the possibility of a direct effect of light upon the permeability of the cell wall and protoplasm to CO_2 cannot be left out of account.

(*i*) *The Relation between Stomatal Capacity for Flow of Air and Stomatal Capacity for Diffusion.*

Up to this point the observed parallelism between porometer rates and assimilation rates, and the relatively consistent relation which these rates bear to one another when results for the same period of the year are considered, have been taken as good evidence that the observed variations in assimilation were

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*** Experiments LXI and LXII lasted only 7 hours; consequently, in drawing the freehand curves for fig. 22, account has been taken of the first three readings only (representing the first 6 hours) of Experiments LVI and LVII.**

largely occasioned by variations in stomatal opening. There remains the question whether the observed changes in porometer rates do, in fact, indicate changes in stomatal opening, of the order required to produce the observed changes in rate of uptake of CO_2. Fortunately, the type of porometer employed and the care taken to standardise the method (see *ante*, pp. 473–474) render it possible to carry the investigation some steps at least in the direction of answering this question.

It is first necessary to reduce the observed rates of air-flow and of diffusion to absolute dimensional units. The dimensions will be expressed in μ units.

1. *Rates of Diffusion.*—The A.A./CO_2 ratio is the rate of uptake of CO_2 by the leaf for a partial pressure of CO_2 of 0·01 atmosphere. The rate of diffusion of CO_2 along a tube of radius, r, and length, l, for a difference in pressure of CO_2 of 0·01 of an atmosphere maintained between the two ends is (using the constant established by Brown and Escombe (4)):—

$$\frac{3600 \times 0 \cdot 145}{100} \times \frac{\pi r^2}{l} \text{ c.c. per hour} = 5 \cdot 22 \times \frac{\pi r^2}{l}$$

(r and l being in centimetre units). Hence,

$$\frac{\text{A.A.}/CO_2 \text{ ratio}}{50 \times \text{N}} = 5 \cdot 22 \times \frac{\pi r^2}{l},$$

where N is the number of stomata per square centimetre and $\pi r^2/l$ represents the dimensions of a tube offering the same resistance to diffusion as is offered by the leaf *per stoma* to the uptake of CO_2. Hence,

$$\frac{r^2}{l} = \text{A.A.}/CO_2 \text{ ratio} \div (5 \cdot 22 \times \pi \times 50 \times \text{N}) \text{ in centimetre units,}$$

or

$$\frac{r^2}{l} = \text{A.A.}/CO_2 \text{ ratio} \times \frac{100}{8 \cdot 2 \times \text{N}} \text{ in } \mu \text{ units.}$$

The dimension r^2/l will be used as the measure of diffusive capacity per stoma.

2. *Rates of Air Flow.*—Flow of air in a tube having the dimensions r^2/l will be proportional to r^4/l, so that, for any observed rate of air-flow *per stoma*, a value r^4/l may be calculated, which will represent the dimensions of a tube offering the same resistance to air-flow as is offered *per stoma* in the observed case. The porometer rate, which is the square root of the rate of flow of air, is thus expressed by the dimensions $\sqrt{(r^4/l)}$.

With a standard capillary tube of known dimensions ($r^4/l = 10^3/3 \cdot 17$ in μ units) the observed time of flow, under the standard conditions for the flow meter, was 18 seconds, corresponding to a porometer rate of $\sqrt{(10^4/18)}$. Hence, for an observed rate of flow of air, P^2 (where P = porometer rate), the dimension r^4/l is $\frac{10^3}{3\cdot 17} \times \frac{P^2}{y} \times \frac{18}{10^4}$, where y = number of stomata per porometer area. Hence $\sqrt{(r^4/l)}$ (square root of capacity for flow *per stoma*) $= P \times \sqrt{\frac{1\cdot 8}{3\cdot 17 y}}$ in μ units.

As already explained, the porometer rates have been so calculated as to allow for non-stomatal resistances in the path of flow (p. 492). The values obtained for square root of capacity for air-flow *per stoma* need, therefore, no further correction. The values obtained for diffusive capacity *per stoma* are, however, only directly valid when the other (non-stomatal) resistances in the path of CO_2 uptake are negligibly small. For the purpose, therefore, of comparing the observed relation between air-flow through the stoma with theoretical expectation, it is necessary to eliminate the non-stomatal resistances in the path of CO_2 uptake. This may be done approximately by the use of the equation already discussed (p. 496)—A.A./CO_2 ratio $= k/(L + S)$, where S is the stomatal resistance and L is the sum of the remaining resistances of the leaf. Used in the form A.A./CO_2 ratio $= \frac{1}{k_1 + k_2/P}$, where P is the porometer rate, this enables us to calculate the relation between the A.A./CO_2 ratio and porometer rate when the remaining resistances are zero, *i.e.*, A.A./CO_2 ratio $= P/k_2$.

In fig. 23 (p. 513) are shown the calculated curves, of the form A.A./CO_2 ratio $= \frac{1}{k_1 + k_2/P}$, for Experiments LI and LII and for Experiment CXI; these are plotted as the dimensions $\sqrt{(r^4/l)}$ for porometer rate and r^2/l for uptake of CO_2. The broken lines show the direct relation between $\sqrt{(r^4/l)}$ and r^2/l calculated from these curves, *i.e.*, what corresponds to P/k_2.

The continuous line on the left of the figure shows the theoretical relation for a stoma of circular cross-section, and 12 μ in length, end effects both for diffusion and for flow being taken into account. (12 μ is the average depth of the stoma of the Cherry Laurel leaves used in these experiments.) The calculated relation for a stoma of circular cross-section with sloping sides would lie slightly above that for a straight-sided stoma. That for a stoma of elliptical cross-section would lie considerably above both these curves. A thin broken line shows the experimental relation which has been observed in experiments on diffusion with *Vicia Faba*, the mean pore length in that case being 15 μ.*

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* Unpublished work by G. E. Briggs and E. J. Maskell.

It is clear that, while the estimated relation in the case of the Cherry Laurel leaves is very close to one observed in the case of leaves of *Vioa Faba*, and is of the same order as the relation expected for a circular stoma, yet it does fall below even the minimum expectation. (The observed diffusive capacity is 50 to 60 per cent. of the minimum expectation.) The theoretical curve is, of course, based on the assumption that all the stomata are of the same size, shape and degree of opening. Actually this is far from being the case; it has been found that the standard deviation of the pore area of a group of stomata may, especially for a small mean pore area, reach a value 50 to 60 per cent. of the mean. Now, since diffusion is approximately proportional* to pore area, and air flow approximately proportional to the square of the area, the mean observed capacity for flow will exceed that theoretically corresponding to the mean observed capacity for diffusion, in the ratio $\frac{\bar{a}^2 + \sigma^2}{\bar{a}^2}$, where $\bar{a}$ is the mean pore area and σ is the standard deviation of pore area. (If eccentricity of pore in cross-section has to be taken into account, the divergence becomes greater still.)

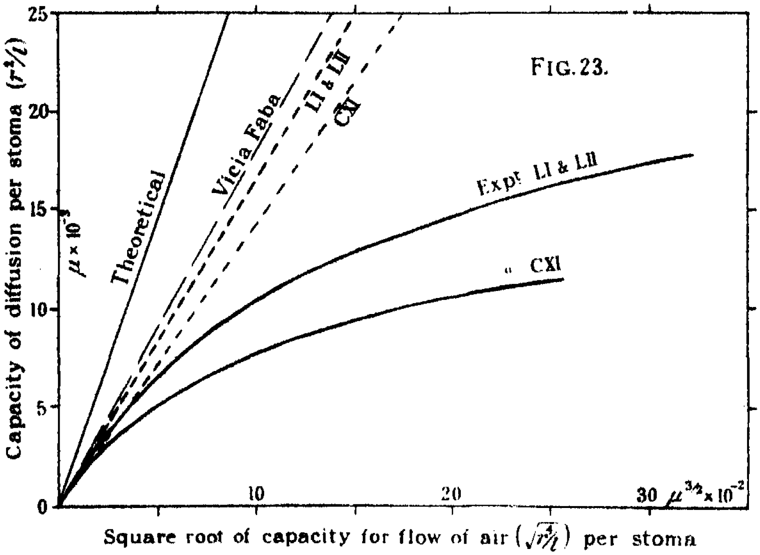

FIG. 23.—Relation between air-flow per stoma and diffusion per stoma (for explanation see text, p. 510, *et seq.*).

· In addition, we have here the possibility suggested earlier (p. 496) that the form of analysis employed neglects any increase in the equivalent resistances in the photochemical and chemical phases of photosynthesis as the stoma opens, and the concentration of CO_2 outside the chloroplast surface increases. Correction for this factor would have the effect of steepening the slope of the curve relating the experimental values of $\sqrt{(r^4/l)}$ and r^2/l, but to what extent it is impossible to estimate in the absence of a deeper knowledge of the dynamics of photosynthesis than we at present possess.*

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* Assuming the depth of the stoma to be greater than its width, which will be true for small stomatal openings.

On the whole, considering the confirmation afforded by the Vicia results, it seems reasonable to regard the stomatal changes, recorded by means of the porometer in these experiments, as of about the order adequate to produce the observed changes in apparent assimilation.

Before leaving the subject of gaseous diffusion in relation to assimilation, it may be appropriate to consider one of the non-stomatal resistances in the path of CO_2 uptake—that of the air diffusion path up to the surface of the leaf (*i.e.*, of the " shell " over the leaf surface)—concerning which it is possible to make some approximate calculations. This resistance cannot be greater than for a leaf of about this size, in the relatively still air of a room. This, on the basis of Renner's experiments (14), would make the " effective length " of the shell 1 cm. As the total depth of the leaf chamber is less than 1 cm., this estimate is much too great, but it will serve for calculation. If, on the other hand, we regard the conditions as those of " a room with little draught,"† the value indicated is about 0·2 cm. The total resistance offered by the leaf to uptake of CO_2 may be calculated as follows :—

The diffusion of CO_2 across an area of 50 sq. cm. and along a length of 1 cm. for a difference of pressure of 0·01 atmosphere will be $\frac{50 \times 3600 \times 0{\cdot}145}{100}$ c.c. per hour = 261 c.c. CO_2. Hence the resistance offered by the leaf is, in centimetres, $\frac{261}{\text{A.A.}/CO_2 \text{ ratio}}$, since the A.A./$CO_2$ ratio is the uptake for 1 hour, for 50 sq. cm. of leaf surface, for 1 per cent. CO_2. From the freehand curves for Experiments LI and LII the maximal A.A./CO_2 ratio would appear to be about 30, corresponding to a resistance of 8·7 cm. (The maximal A.A./CO_2 ratio from the calculated equation (p. 496) is higher, 37·7, giving a resistance of 6·92 cm.) Thus the resistance in the air path up to the leaf surface, which we may put at between 0·2 and 1·0 cm., can form but a small part of the total resistance.

The greater part of the non-stomatal resistance must lie in the liquid diffusion phase and in the photochemical and chemical phases of the process. In the absence of any data for the average area of cell surface associated with a single stoma of the Cherry Laurel leaf and for the average length of the liquid diffusion path from cell wall to chloroplast surface, an estimation of the resistance in this phase cannot be made. A thorough investigation of the structure of the Cherry Laurel leaf from this point of view is much to be desired, but it could not be carried out in the time at the writer's disposal.

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* An approximate solution is offered later (p. 521, *et seq.*).

† See Jeffreys (9).

(*j*) *On the Effect of "stirring" the Air in the Plant Chamber.*

These calculations make the air path resistance between 3 and 14 per cent. of the total. During the course of the experimental work a large number of experiments were carried out, in which the air in the leaf chamber was violently stirred or agitated, in order to determine whether any effect on apparent assimilation could be observed. Periods of "wind" were alternated with periods of "still" air, in which only the normal rate of current was maintained. The effect observed was very small, the maximum observed effect being less than 10 per cent. and the average effect less than 5 per cent. This accords with the calculations made above.

(*k*) *Summary of Experimental Results.*

Certain general features of the results described in this and in the preceding paper may be briefly summed up here.

It has been shown that for the Cherry Laurel leaf there is not, for any one external concentration of CO_2, one definite rate of apparent assimilation as appeared to be the case for Elodea (Blackman & Smith, 1910 (2)); nor can the rate of assimilation be defined by defining the light intensity in addition to the external CO_2 concentration (as appears to be the case for the experiments of Lundegårdh (11), Warburg (15) and Harder (7)). The variations observed in rate of uptake of CO_2 have been shown to be due to variations both in stomatal resistance and in the remaining resistances of the leaf.

The stomatal resistance has been shown to depend upon—

(1) the season of the year (the range of stomatal opening at constant light intensity being different for different months);

(2) the time of the day (evidenced by the diurnal rhythm of stomatal opening at constant light intensity);

(3) the time elapsed from the beginning of the experiment;

(4) the intensity of the illumination;

(5) the previous history of the leaf (*cf.* experiments on "direct" leaves as compared with "24-hour" leaves).

(6) the previous moisture conditions of the plant (experiments on leaves during the drought of 1921).

The non-stomatal resistance has been shown to depend upon—

(*a*) the time from the beginning of the experiment (this effect is also a function of season (p. 500) and of the previous conditions of the plant (p. 502);

(*b*) the intensity of the illumination (pp. 509–10);

(*c*) the moisture conditions of the leaf (experiments on "slashed" leaves (12)).

Fig. 24, in which are collected the freehand curves for adjusted porometer rate and A.A./CO_2 ratio of six groups of experiments, shows that the non-stomatal resistance depends also upon the season of the year, and upon the previous moisture conditions of the plant. The relative magnitude of this resistance will roughly be inversely proportional to the value of the A.A./CO_2 ratio at a porometer rate of ∞, *i.e.*, to the apparent maxima to which the curves tend. Comparing curve 1 (December, 1920) with curve 3 (July, 1922), it will be noted that the winter values lie well above the summer values. The same is true for curves 4 (December, 1921, and January, 1922) and 5 (June to October, 1921), but both these curves, which are for leaves developing during the drought of 1921, lie below the corresponding curves for the more normal years 1920 and 1922.

Curve 2, for late January and February, 1920, differs from that for December, 1920, in its lower portion (see earlier, p. 497), but tends to the same maximum, while that for April, 1921, curve 7, though of the same form as curve 2, lies definitely below both curve 1 and curve 2.*

These seasonal variations show a remarkable similarity to the seasonal variations in photosynthetic activity, measured with temperature or light limiting, described by Miss Matthaei (13) for Cherry Laurel. She found high values January to March, low values in April (the value for unit light in April is about 30 per cent. below that for unit light in January, February and March), and low values also in the summer. The change between October and November in the light limited values of apparent assimilation in this series of experiments has already been noted (12). In February, 1921, the light limited value

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* To avoid complicating the picture only the upper parts of curves 2 and 7 are given.

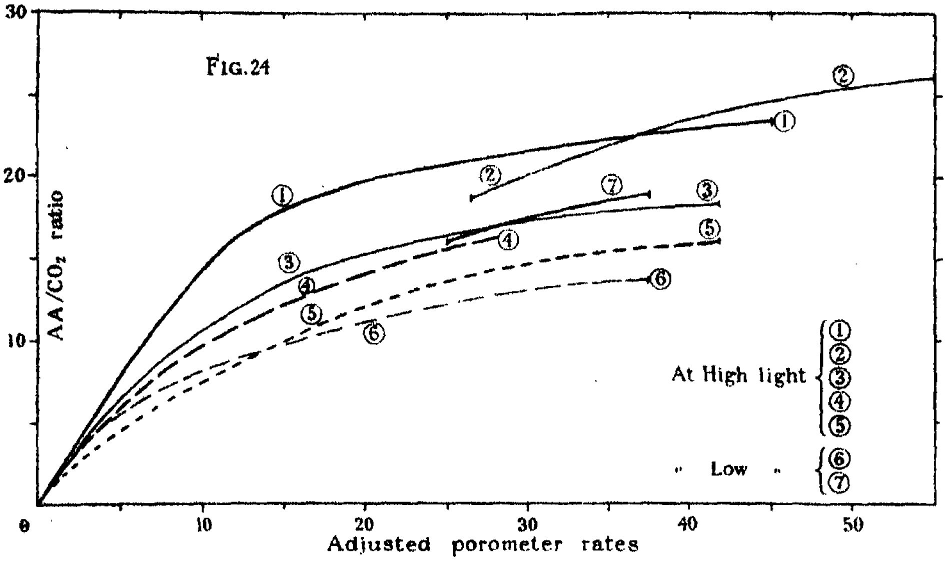

FIG. 24.—Relation of assimilation rate to porometer rate at different seasons.

Curve 1.—Experiments LI and LII, December 6–9, 1920.

Curve 2.—Experiments LVI, LXI and LXII, January 20 and February 18–22, 1921.

Curve 3.—Experiments CXI, July, 1922.

Curve 4.—Experiments CVII and CVIII, December 9, 1921, and January 4, 1922.

Curve 5.—Experiments XCIV, CI and CIII, June 17, October 4, and October 7, 1921.

Curve 6.—Experiments LVII, LXI and LXII, January 24, and February 18–22, 1921, at *low light.*

Curve 7.—Experiment LXXIV, April 26–27, 1921. *N.B.*—This curve is erroneously marked as "at Low Light" in the diagram, it should be "at High Light."

is still at the level found in November, 1920, but in July, 1922, it is down to the summer level again. This change from summer to winter agrees with Miss Matthaei's observations.

This effect of season upon the non-stomatal resistance thus appears to be attributable to seasonal changes in the "resistance" in the photochemical and chemical phases of photosynthesis. Such changes have been described by Briggs, 1922 (3), as changes in amount of active chloroplast surface. The evidence here presented shows that seasonal variations in this factor affect assimilation when CO_2 is limiting as well as when light and temperature are limiting—a result predicted by Briggs* in the paper just mentioned. Data are not unfortunately available for the light limit during the drought (curves 4 and 5). It seems probable that in this case part of the observed depression in assimilation may be due to a change in the resistances in the liquid diffusion phase.

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* Briggs (*loc. cit.*), p. 33.

With the apparent assimilation for constant external CO_2 concentration thus affected by such a galaxy of internal and external factors, in what sense can the external concentration be said to be *limiting* the rate of assimilation? Certainly not in the sense in which the term applied to the Elodea results of Blackman and Smith, nor in the sense in which the term can be applied to light or temperature.

There appears to be for any given light or temperature a maximal assimilation which cannot be increased by increase in any other external factor. (The value of the maximal assimilation per *unit area of leaf* would be determined by the amount of "reactive chloroplast surface" per unit area of leaf.) Temperature and light will, therefore, set definite limits to the rate of assimilation, limits which can for moderate intensities of light and of temperature be ascertained with reasonable accuracy. The only limit in this sense which could be set by the external CO_2 concentration would, for any given leaf, be reached when the stomata were at their maximal opening, the wind conditions such that the concentration of the CO_2 immediately over the leaf surface was practically the same as that in the external air, the resistance to diffusion through cell wall and protoplasm no greater than that for an equal diffusion path of water, and the concentration at the chloroplast surface approximately zero.

This distinction, between CO_2 on the one hand, and light and temperature on the other, has been indicated in this paper by the use of inverted commas for the term "limiting" when applied to CO_2. There is, however, a sense in which the term may be useful. CO_2 may be said to be "limiting" the rate of apparent assimilation when a small change in the CO_2 concentration, other factors being constant, produces an approximately proportionate change in apparent assimilation. For land leaves this relation appears, from the work of Brown and Escombe (5) and Lundegårdh (11), to hold over a range of CO_2 concentrations very much exceeding that of the normal atmosphere, and apparently up to an assimilation rate approaching the temperature or light limit. The present experiments, so far as they offer evidence in this direction, confirm these conclusions.

Retaining the term "CO_2 limiting" in this sense, it may be convenient here to make as precise as possible the form in which an analysis of the rate of apparent assimilation of the "CO_2 limited" leaf can in the writer's opinion be usefully made. The rate of assimilation under these conditions is regarded as being determined by the external CO_2 concentration and a set of resistances between "source" and "sink." Thus

$$\text{Apparent Assimilation} = k \frac{\text{external } CO_2 \text{ concentration}}{r_1 + r_2 + r_3 + r_4 + r_{\text{L.T.}}},$$

where r_1 is the resistance to diffusion in the air path up to the leaf surface, r_2 that of the stomata, r_3 that of the intercellular space system, r_4 that of the path of liquid diffusion, and $r_{\text{L.T.}}$ the equivalent "resistances" in the photochemical and chemical phases of the process of photosynthesis; all the resistances being expressed per unit area. These last resistances, $r_{\text{L.T.}}$, will be a function of light and temperature and of those internal factors which affect assimilation when light and temperature are limiting (p. 518). At concentrations of CO_2 such that the assimilation approaches the light or temperature limits, these last resistances must be a function of CO_2 concentration also, but, for the region in which approximate proportionality between assimilation and external concentration of CO_2 holds, this complication may obviously be neglected.

The effect of change in any external or internal factor, other than CO_2, upon the rate of assimilation under these conditions will depend upon what fraction the resistance or resistances affected by the external factor are of the total resistance. Thus at "high" light intensity and adequate temperature, when $r_{\text{L.T.}}$ is small, a given change in stomatal opening will have a bigger effect than at "low" light intensity, and similarly the effect of change in light intensity apart from its effect on the stoma will be greater at wide stomatal opening than at narrow.

SECTION II.—THE INTERACTION OF LIMITING FACTORS—A DISCUSSION.

(a) *Recent Work.*

Two aspects of what is understood as the theory of limiting factors have been the subject of papers appearing while this work was in progress:—

(1) The form of the curves relating concentration of CO_2 or light intensity to rate of assimilation; and

(2) The possibility of increasing the rate of assimilation by increasing the intensity of other factors than that which is held to be limiting.

The conception of assimilation developed in the last section will be used as a basis for the discussion of these results.

Lundegårdh (11) has studied the relation of apparent assimilation to increasing intensity of light for sun leaves (Nasturtium), and for shade leaves (Oxalis). The relation for the shade leaf is regarded by Lundegårdh as approximating to the "limiting factor curve" of Blackman and Smith, that for the sun leaf as approximating to the relation established by Warburg (15). Differences in diffusion relationships are offered as an explanation of the difference between the two curves. Lundegårdh interprets the rising limb of the curves as light-limited, and the horizontal, or approximately horizontal, portion, as CO_2 limited; and is able to show that, even in the "light limited" region, the assimilation can still be increased by increase in CO_2 concentration. But the rising limb of the curve cannot be accepted as light limited in the sense used by Blackman and Smith and retained in this paper (p. 518). The true light limited values of assimilation are only reached at much higher CO_2 concentrations.

Again, the values of assimilation used in Lundegårdh's curves are not simple assimilation values, but have been corrected to a CO_2 concentration of 0·57 mg. per litre. They are, in fact, A.A./CO_2 ratios and, moreover, there can be no doubt that their increase with increased intensity of illumination is, in part, to be ascribed to the effect of light on stomatal opening—an effect which would in itself give curves of the form shown. It is significant that according to Lundegårdh the stomata of Oxalis, a shade plant, may be found fully open at light intensities above "one twelfth," while in deeper shade they are only one-half to one-quarter open; for his light-assimilation curve reaches its maximum value at about "one-tenth" light. In the case of sun leaves, maximal stomatal opening may with reason be supposed to be reached only at higher light intensities.

It is not surprising, therefore, to find that Lundegårdh's "CO_2 curves" (relating apparent assimilation and CO_2 concentration), at various constant light intensities, are quite different in form from his "light curves." The "CO_2 curves" appear to consist of an approximately linear limb, the "CO_2 limited" region, and an approximately horizontal limb, the light limited region, connected by a quite narrow transitional region. It seems clear in this case that interaction between CO_2 and light in the photosynthetic process itself can be practically neglected until the light limit is almost reached. On the view suggested here, the steeper slope of the "CO_2 curves" at the higher light intensities would be due to a wider mean stomatal opening.

The case is quite different in the work of Harder (7), and of Warburg (15), where the "CO_2 curves" as well as the "light curves" are of form shown by the light curve of Lundegårdh's sun leaf, *i.e.*, the curve passes *gradually* from a region of rapid increase with increase in the variable factor to a region in which increase with increase in the variable is zero. These experiments are for water plants in carbonate-bicarbonate solutions, and it is difficult to compare the conditions with those of a land leaf. It seems clear, however, that in these cases the effect of the CO_2 concentration at the chloroplast surface upon the "resistances" of the photochemical and chemical phases cannot at any point be left out of account.

Any analysis of the dynamics of photosynthesis aiming at completeness must necessarily provide for interaction between CO_2 and light of the type found by Harder and Warburg. The position may roughly be stated as follows: The curve relating CO_2 and assimilation or light and assimilation may be regarded as approximating in form to an oblique hyperbola. When the "corner" is sharp, a statement of the two asymptotes to the curve will describe the curve for all practical purposes (this is the typical "limiting factor relationship" of Blackman and Smith's Elodea results, and of Lundegårdh's CO_2 curves). When the corner is more gradual a statement of the two asymptotes—representing the analysis in terms of limiting factors—will be inadequate even for the empirical description of the phenomena, and we shall need the full equation to the curve. For the complete description of the phenomena we shall in any case need the full equation.

In the case of the land leaf, interaction between light and CO_2 of the type found by Lundegårdh may in part be attributed to the effect of light upon stomatal opening. In the case of Cherry Laurel we have evidence that in addition to an effect of light on stomatal opening there is an effect on the non-stomatal resistances in the path of CO_2 uptake; and seasonal changes in the "photosynthetic activity" of the leaf (as measured by the seasonal changes in *light limited* assimilation values) have a parallel effect (*cf.* curves 1, 3, and 6, fig. 24).

An attempt will be made, therefore, to take into account this interaction between light and CO_2 in the case of Cherry Laurel, and to combine this with some consideration of the resistances in the diffusion phase of photosynthesis. It is realised that the solution offered must be far from complete, in particular because the effect of temperature and the problem of the chemical or "dark" phase of photosynthesis must be neglected.

(*b*) *Theoretical Scheme for the Case of the Land Leaf.*

For the present case, in which the assimilation is well below the temperature limit, we may adopt Warburg's picture of the photochemical phase, as a simple expression of a possible type of relationship between CO_2 and light *at the chloroplast surface*. On this view the rate of assimilation is *proportional* to $\frac{L \times C_S}{C_S + K_L}$, where L is light intensity, C_S is concentration of CO_2 at the chloroplast surface, and K_L is the ratio of the velocity constant for the dissociation of some photochemical product (the rate of formation of which is proportional to L) to the velocity constant for the combination of this photochemical product with CO_2.

If now we express light intensity in terms of the light limited values of real assimilation we have Real Assimilation, y, as :—$y = \frac{L \times C_S}{C_S + K_L}$. Assuming now, for simplicity, that we may neglect any resistance in the diffusion path of the respiratory CO_2 up to the chloroplast surface (or that this path is short compared with the path to the outside of the leaf) we have :—Apparent Assimilation $= \frac{C - C_S}{D} = \frac{L \times C_S}{C_S + K_L} - R$, where C is external concentration of CO_2 and D is the total resistance in the diffusion path of CO_2, from outside the leaf up to the chloroplast surface. Hence Real Assimilation is $y = \frac{L \times C_S}{C_S + K_L} = \frac{C - C_S}{D} + R$, from which

$$(a) \quad y = \frac{C}{D} - \frac{K_L}{D} \cdot \frac{y}{(L - y)} + R.$$

For the curve relating y to increase of C, we have*

$$\frac{dy}{dC} = \frac{1}{D}\left(\frac{1}{1 + \frac{K_L}{D}\left(\frac{L}{(L-y)^2}\right)}\right).$$

When $y = L$, *i.e.*, when the light limit is reached, then $dy/dC = 0$; or, $y = L$, forms the horizontal asymptote of the curve relating y and C. When $y = \pm \infty$, then $dy/dC = 1/D$, this being the slope of the asymptote to the ascending limb of the curve.

For any given fraction, y/L, of the maximal assimilation at any given light intensity, the slope dy/dC of the curve relating assimilation to CO_2 concentration will be relatively less than the asymptotic value, $1/D$, according as

$$\frac{1}{1 + \frac{K_L}{D}\left(\frac{L}{(L-y)^2}\right)}$$

is less than unity, *i.e.*, according as D or L are relatively small or as K_L is relatively great. That is to say, the "corner" of the "CO_2 curve" will be sharp for relatively high values of D (the resistance to diffusion of CO_2 into the cell) or of L (the light intensity) and sharp also for relatively low values of K_L (low values of K_L representing a high efficiency of the photochemical mechanism at the chloroplast surface).

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* Following the usual practice it is assumed that rate of respiration is constant, apart from temperature fluctuations, which are left out of account here.

Similarly, for the curve relating assimilation and light intensity,

$$(b) \quad y = L \times \frac{\frac{C}{D} + R - y}{\frac{C}{D} + R - y + \frac{K_L}{D}},$$

from which

$$\frac{dy}{dL} = \frac{\frac{C}{D} + R - y}{\frac{C}{D} + R - y + \frac{K_L}{D}}.$$

When $y = \frac{C}{D} + R$, then $\frac{dy}{dL} = 0$; or $y = \frac{C}{D} + R$ is the horizontal asymptote of the curve. When $y = \pm \infty$, then $dy/dL = 1$; this is therefore the slope of the asymptote to the ascending limb of the curve. Similarly, for $C = \infty$ $d_y/d_L = 1$.

Thus for any given fraction of the maximal possible assimilation, $C/D + R$, at any given CO_2 concentration, d_y/d_L falls below unity according as D, C or R are relatively small or as K_L is relatively great. That is to say, the "corner" of the curve relating light and assimilation will be sharp for relatively high and gradual for relatively low values of D (the resistance to diffusion of CO_2 into the cell), or of C (the external CO_2 concentration), or of R (the respiration). A change in the relative value of K_L will operate in the reverse direction.

It seems clear that, if this equation forms any approximation to the real relation between the factors controlling photosynthesis, the divergences found in the form of the curves relating assimilation to CO_2, or to light, represent variations due largely to different relative values of the resistances in the diffusion and photochemical phases of the process. The Elodea result would correspond to a set of conditions with a high resistance to diffusion, or a high efficiency of light at the chloroplast surface or a high light intensity, while Harder's results would correspond to a set of conditions with a relatively low resistance to diffusion or a low efficiency of light at the chloroplast surface or both of these.

The results may be illustrated by the following approximate solution of the case of the Cherry Laurel leaf in terms of the suggested equation—Real Assimilation $= \frac{C - C_S}{D} + R = \frac{L \times C_S}{C_S + K_L}$. The resistances in the diffusion phase may be expressed as centimetres in an equivalent air diffusion path (*v. ante*, p. 514): C (the external CO_2 concentration) therefore becomes $261 \times$ mean CO_2 per cent. The total diffusion resistance, D, is subdivided into a stomatal resistance, D_S (estimated as $k \times 1/P$, where $P =$ porometer rate), and the remaining resistances of the diffusion path, D_R.

Light intensity, L, is expressed in terms of the light-limited real assimilation values. (For the *winter* season, 1920–1921, these are: "high" light, 6·95 c.c. CO_2; "low" light, 2·9 c.c. CO_2, per 50 sq. cm. per hour. For the *summer* of 1920 they are: "high" light, 6·2 c.c.; "low" light, 2·6 c.c.) The effect both of change in light intensity and of seasonal changes in photosynthetic efficiency (*v.* p. 517) is thus expressed by a change in the value L. Respiration, R, will be slightly different at the two light intensities owing to the difference in leaf temperature. At "high" light it is taken as 0·4 c.c., at "low" light as 0·3 c.c.

The case taken for solution is that represented by the experiments at "high" and at "low" light in the winter of 1920–1921—Experiments LI, LII, LVI, LXI, LXII and LVII (curves 1, 2 and 6, fig. 25). In order to obtain a maximum estimate for the interaction between light and CO_2 in the reactions at the chloroplast surface, it has been assumed that the *whole* of the observed effect of light upon apparent assimilation, at constant stomatal opening, is due to the effect of light intensity upon the "resistances" in the photochemical phase (*v. ante*, p. 510) and that there is no effect upon the resistances in the liquid diffusion phase. This gives us a maximum value for K_L. On this assumption the values of the constants obtained by trial are $D_R = 6$, $D_S = 120 \div P$,[*] $K_L = 6$, so that, for "high" light, Apparent Assimilation is

$$\frac{261 \times CO_2 \text{ per cent.} - C_S}{6 + 120/P} = \frac{6{\cdot}95\, C_S}{C_S + 6} - 0{\cdot}4,$$

and for "low" light

$$A.A. = \frac{261 \times CO_2 \text{ per cent.} - C_S}{6 + 120/P} = \frac{2{\cdot}9\, C_S}{C_S + 6} - 0{\cdot}3.$$

Theoretical CO_2 curves using these constants will be found in fig. 26 (p. 526), but we may consider first the theoretical curves relating porometer rate and A.A./CO_2 ratio, since the experimental curves of this type have been so largely used in developing the present series of data. These curves are shown in fig. 25. Curve 1 is for 0·15 per cent. mean CO_2 concentration, and the "high" light, and may be compared with the experimental (freehand) curves 1A, for Experiments LI and LII, and 1B, for Experiments LVI, LXI and LXII. For porometer rates above 10, the mean CO_2 concentration for curve 1A was 0·17 per cent., and for curve 1B, 0·15 per cent.

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[*] This would bring the relation between stomatal capacity for flow of air and stomatal capacity for diffusion about midway between the theoretical curve, fig. 23, and the relation previously calculated (*v.* p. 511).

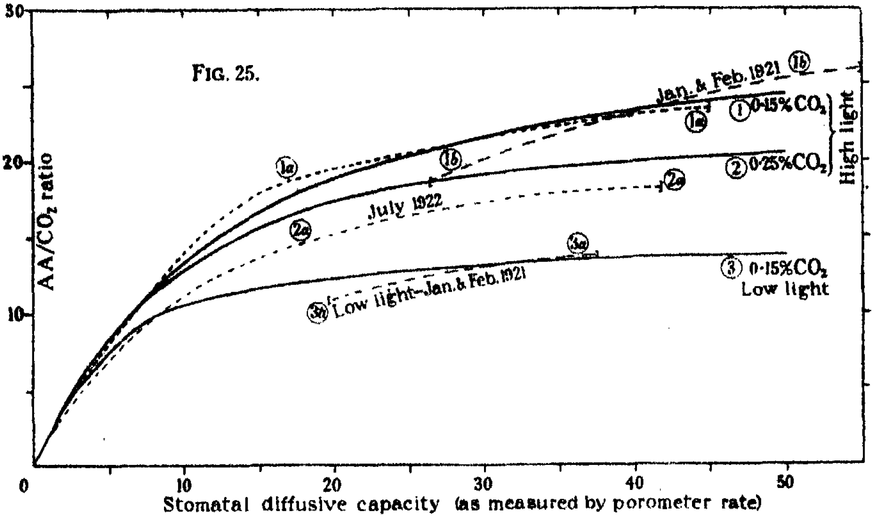


FIG. 25.

FIG. 25.—Theoretical relation between stomatal diffusive capacity and assimilation (mean CO_2 per cent. and light constant). Theoretical curves (1, 2 and 3) are shown by the continuous lines. Experimental curves (1A, 1B, 2A and 3A) by broken lines.

Curve 1.—"High" light, mean CO_2 = 0·15 per cent.

Curve 2.—"High" light, mean CO_2 = 0·25 per cent.

Curve 3.—"Low" light, mean CO_2 = 0·15 per cent.

Curve 1A, December, 1920, Experiments LI and LII. "High" light, mean CO_2 (for porometer rates above 10) = 0·17 per cent.

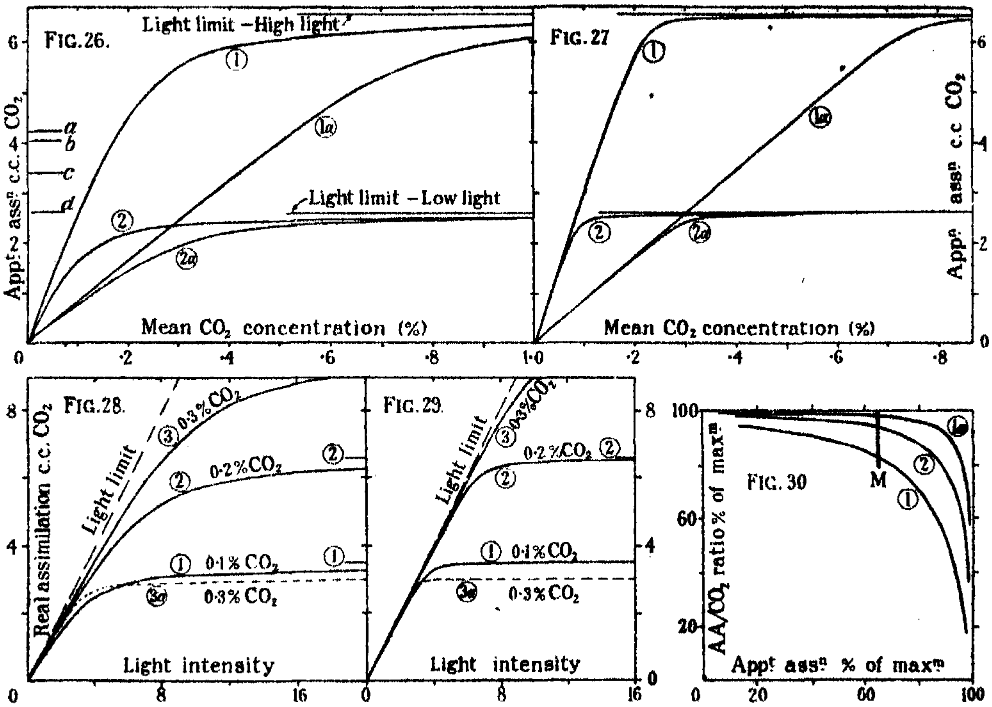
Curve 1B.—January and February, 1921, Experiments LVI, LXI and LXII. "High" light, mean CO_2 (porometer rate < 10) = 0·15 per cent.

Curve 2A.—July, 1922, Experiment CXI. "High" light, mean CO_2 (porometer rate < 10) = 0·25 per cent.

Curve 3A.—January and February, 1921, Experiments LVII, LXI and LXII. "Low" light; mean CO_2 (porometer rate < 10) = 0·15 per cent.

For the theoretical equation, see text, p. 524.

It will be seen that the theoretical curve 1 is closer to the experimental curve 1A than the curve previously calculated (pp. 496–7) is. Both curves rise sharply and then flatten out. At high porometer rates the A.A./CO_2 ratio is affected by the CO_2 concentration, so that the theoretical curve for 0·25 per cent. CO_2, curve 2, lies, for high porometer rates, below that for 0·15 per cent. CO_2.



FIGS. 26, 27, 28, 29, 30.—Theoretical light-assimilation and CO_2-assimilation curves for Cherry Laurel.

FIG. 26.—Relation of apparent assimilation to mean CO_2 concentration.

Curve 1.—" High " light, porometer rate = 50.
Curve 1A.—" High " light, porometer rate = 5.
Curve 2.—" Low " light, porometer rate = 50.
Curve 2A.—" Low " light, porometer rate = 5.

The asymptotes of the curves, $CO_2 = \infty$, are shown by the horizontal lines on the right of the figure. The horizontal lines *a*, *b*, *c*, *d* on the left mark the highest levels of apparent assimilation reached in the experiments with CO_2 " limiting " discussed earlier.

a. Experiment CXI, July, 1922.
b. Experiments LI, LII and LVI, December, 1920, and January, 1921.
c. Experiments XCIV, CI and CIII, June and October, 1921.
d. Experiments CVII and CVIII, December, 1921, and January, 1922.

FIG. 27.—As fig. 26, but the constant K_L (see text) in the photochemical phase reduced from 6 to 0·5.

FIG. 28.—Relation of real assimilation to light intensity. Light intensity is measured in terms of light limited assimilation values.

Curves 1, 2 and 3 are for 0·1, 0·2 and 0·3 per cent. mean CO_2, and porometer rate = 50.

FIG. 28—(continued).

Curve 3A (broken line) is for 0·3 per cent. CO_2 and porometer rate = 5. The position of the asymptotes (at L = ∞) is indicated by the levels *1*, *2*, on the right of the figure.

FIG. 29.—As for fig. 28, but the constant K_L in the photochemical phase reduced from 6 to 0·5.

FIG. 30.—Theoretical relation between A.A./CO_2 ratio at "high" light and level of apparent assimilation at which it is measured Both A.A./CO_2 ratio and apparent assimilation are expressed as a percentage of their maximum value.

Curve 1.—Porometer rate = 50.

Curve 2.—Porometer rate = 5; and for both curves K_L = 6.

Curve 1A.—Porometer rate = 50, but K_L = 0·5.

The line M marks the position of the level, *a*, in fig. 26, so that the experimentally used values of the A.A./CO_2 ratios lie almost entirely to the left of M and above curve 1.

Experiment CXI, in the summer of 1922 (curve 2A), was at a mean CO_2 concentration (for porometer rates above 10) of 0·25 per cent. It will be seen that it lies well below expectation, so that, although part of the depression noted earlier (p. 516) in this summer curve, as compared with the winter curve, 1A, may be due to the higher mean CO_2 concentration, part cannot be so explained, but is probably due, as was suggested earlier, to seasonal changes in photosynthetic activity. Calculation from the level of the curve would suggest a light-limited assimilation value of about 5·8 c.c. as against 6·95 c.c. for the winter season of 1920–1921.

Curve 3 is for "low" light intensity and 0·15 per cent. CO_2, and, with curve 1, represents the effect of change in light intensity observed in the experimental curves 1B and 3A (p. 526). The mean CO_2 per cent. for porometer rates above 10, for curve 3A, was 0·153 per cent.

It will be seen that the general results obtained from the consideration of the experimental curves can be reproduced in the theoretical curves, and that the types of interaction found between apparent assimilation and stomatal opening, seasonal changes in photosynthetic activity, and light intensity, follow naturally from the theoretical formula.

In figs. 26 and 28 (p. 526) are shown the theoretical curves, relating assimilation to CO_2 concentration and to light intensity, derived from the formula on p. 522, with the constants as calculated for the case of Cherry Laurel. In fig. 26 *Apparent Assimilation* is plotted against external CO_2 concentration. It will be seen that the curves pass approximately through the origin. Curves 1 and 1A are for "high" light, at wide stomatal opening (porometer rate = 50, curve 1) and narrow stomatal opening (porometer rate = 5, curve 1A) respectively. Curves 2 and 2A are for the "low" light intensity at the same stomatal openings. It will be seen that the "corner" of the CO_2 curves is sharper with the "high" light intensity than with the "low," and sharper also at the narrow stomatal opening than at wide. It may be noted that curves 2A and 1 reproduce the type of relationship shown by Lundegårdh's "CO_2 curves" at different light intensities if we assume that the stomatal opening is narrow ($P = 5$) at the "low" light and wide ($P = 50$) at the "high" light.

The horizontal lines on the left of the figure mark the maximal values of apparent assimilation found in those experiments with CO_2 "limiting" which have been used for the earlier analysis of the phenomena, *e.g.*, fig. 24. They show that, even with the widest stomatal opening (curve 1) we have been working over a region of the CO_2 curve which is approximately linear and that the use of the A.A./CO_2 ratio has not seriously distorted the results. The majority of the experimental points will, of course, lie between curves 1A and 1.

An alternative method of expressing this fact is shown in fig. 30, where the theoretical A.A./CO_2 ratio, calculated as a percentage of the maximum possible (*i.e.*, as a per cent. of the slope of the CO_2 curve at zero assimilation) is plotted against the Apparent Assimilation, expressed as a percentage of the possible maximum (the light limit). The vertical line M marks the position of the highest apparent assimilation rate observed with CO_2 "limiting" (level *a* in fig. 26). It will be seen that up to this level the fall in the A.A./CO_2 ratio, as the assimilation increases (with increasing external CO_2 supply), is relatively small, even for the wide stomatal opening (curve 1) and almost negligible for the narrow stomatal opening (curve 2). Thus, at the wide stomatal opening, between an assimilation rate of 2·56 (39 per cent. of the maximum) at 0·1 per cent. CO_2 (the lowest used at this stomatal opening), up to an assimilation rate of 4·2 c.c., or 64 per cent. of the maximum, the A.A./CO_2 ratio falls only from 91·5 per cent. of its maximum to 82·5 per cent. of its maximum; while, for the narrower stomatal opening, starting at the same CO_2 concentration and working up to the same upper level of assimilation, the ratio falls only from 97·5 per cent. of its maximum to 94·8 per cent. While, therefore, the theoretical equation is believed to represent the relations more truly than was possible by confining attention to the A.A./CO_2 ratios, yet the use of this provisional tool, which made possible an economical description of the experimental results, has not seriously distorted their presentation.

Theoretical "light curves" for the constants already used (p. 524) are shown in fig. 28. Stomatal opening and respiration (0·4 c.c.) have been assumed constant at all light intensities and three curves (1, 2 and 3) are given for 0·1 0·2 and 0·3 per cent. CO_2 respectively, at a porometer rate of 50. Curve 3A illustrates, for a porometer rate of 5 and 0·3 per cent. CO_2, the sharp corner obtained when the diffusion resistance becomes relatively great.

In view of the fact that, as noted earlier (p. 524), the value obtained for the constant, K_L, is a maximum, since no allowance was made for a possible effect of light on the resistances in the liquid diffusion phase, it was of interest to calculate "CO_2 curves" and "light curves" for a much smaller value of K_L in order to exhibit the effect of the relative magnitude of this constant.

The curves, shown in figs. 27 and 29, reproduce the conditions for the similar curves in figs. 26 and 28, with the exception that K_L is taken as 0·5 instead of 6·0. The very close approach of this set of curves to the "typical" limiting factor type needs no comment. The very small departure from linearity shown by the "CO_2 curve," at "high" light and wide stomatal opening, for more than 80 per cent. of its length, may be seen from the curve 1A for this case in fig. 30.

(c) *The General Theory of Limiting Factors.*

The outlook of physiologists upon the quantitative relationships between physiological processes and the external factors which affect their rate was, previous to the introduction by Blackman in 1905 (1) of the conception of limiting factors, largely governed by the idea of three cardinal points—a minimum, an optimum and a maximum—characteristic for each external factor in respect of any one process. The conception of limiting factors removed this stumbling block from fundamental physiological research and provided—

(1) A first approximation to a quantitative analysis of the interaction of the external factors controlling a process;

(2) A principle of experimental procedure which enabled the fundamental relationships between any one factor and the rate of the process to be ascertained.

A conspicuous success of the conception and the experimental procedure was the establishment of the fundamental relationship between assimilation and temperature, a success which led the way in the application of the results of physical chemistry to the analysis of physiological processes. The subsequent history of the conception has followed a course unfortunately too common in biological science.

The "first approximation" has been widely misconstrued as a rigid law; and the term "limiting factors" has been borrowed on all hands for the purpose of describing phenomena in quasi-quantitative terms with the minimum of quantitative investigation. The principle of experimental procedure has, on the contrary, been largely neglected.* "The physiologist must take deliberate thought to the other factors, lest surreptitiously one of *them* becomes the real limiting factor to increase in functional activity." It is curious that this caution should have been construed into a permission to leave other factors out of account, except at the point where the rate of the process under investigation ceases to increase with increase of the factor under investigation.

As is inevitable in scientific work, the caution now needs extending to cover a wider range of possibilities, and in the investigation of the functional relationships between any one factor and the rate of the process, an influence upon that relationship of the constant factors must be under suspicion at all ranges of intensity of the variable factor (although in practice this effect of the other factors may be very small at relatively low intensities of the variable factor). Similarly the analysis of the interaction of external factors must take a form determined by the special conditions of the process in question. The particular equation developed in this paper is only a first approximation to such a form and its limitations are fully realised.

As a general picture of the type of interaction between external factors, to which the formulation of the conception of limiting factors drew attention, it may, for some purposes, as in the case discussed in this paper, be convenient to regard the process as determined by a potential and a set of resistances, influenced or determined in part by different external factors. The relative effect of change in any one of these resistances will be a minimum when the remaining resistances are relatively great, and a maximum when the remaining resistances are relatively small; but at all points the whole set of resistances will determine the rate. The chief advantage of this type of picture is that it prevents the neglect of any of the constant factors.

It is impossible, however, to suggest any general picture of more than limited validity. Regarded and used as a clue to the interpretation of the phenomena, the general principle of limiting factors suggested by Blackman in 1905 cannot as yet be replaced.

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* An example of this neglect, which makes it difficult to relate the author's results to other work, is found in a recent paper by Ilјin, 1923 (8), dealing with some of the problems which are dealt with in the present paper. That writer describes experiments on carbon assimilation in which no care is taken to investigate which of the external factors is controlling the rate. The CO_2 per cent. employed is high (about 6 per cent.), and *a priori*, one would expect assimilation under those conditions to be limited by either light or temperature. Yet an observed parallelism between porometer rate and assimilation rate is taken as evidence of a direct causal relationship between variations in stomatal opening and the observed variations in assimilation. Such a relationship could only occur if CO_2 were "limiting," and there is no evidence that this was the case. Nor, in the absence of absolute data with regard to flow of air, can the porometer values given be used to determine whether the stomatal changes were of an order consistent with the writer's conclusions.

Summary.

(1) The experimental work described in this and the preceding communication was begun as an attempt to extend to the case of the land leaf the type of investigation carried out for water plants by Blackman and Smith, on the relation of assimilation to carbon dioxide concentration and other factors, notably light intensity. In particular it was hoped that by taking continuous series of readings of assimilation over a period of 12 to 24 hours, and by changing the external conditions during the period of experiment, the relationships of the various controlling factors might become clear.

(2) At the outset the difficulty is met that, in Cherry Laurel leaves, even under constant illumination, steady rates of assimilation are only maintained when the external CO_2 concentration is so high that light and not CO_2 is limiting the rate. Under these conditions the light limited value of assimilation is maintained steadily for more than 24 hours. With lower concentrations of CO_2, such that the assimilation is below the light limited value, the assimilation falls rapidly, during the afternoon and evening, to very low values, but rises again during the following morning.

(3) Evidence is presented which suggests that this diurnal rhythm of assimilation is due to a diurnal rhythm of stomatal opening.

(4) The pitch or level of this diurnal march of assimilation is higher at high light intensity than at low, and higher also in winter than in summer. It is suggested that these phenomena are due to an effect of light intensity and of season on range of stomatal opening.

(5) The stomatal explanation of these diurnal and seasonal variations in assimilation is tested by means of parallel observations on porometer rate and assimilation rate, and the investigation is extended as a study of the relation between stomatal opening and rate of assimilation.

(6) It is shown that there are diurnal and seasonal rhythms of stomatal opening which run parallel with the diurnal and seasonal rhythms of assimilation rate. An approximate estimate of stomatal diffusive capacity, from the data for air flow per stoma, suggests that the stomatal changes observed are of about the order required to produce the observed changes in assimilation rate.

(7) The form of the relation between stomatal diffusive capacity (as measured by porometer rate) and rate of assimilation suggests that the rate of apparent assimilation (CO_2 "limiting") may usefully be regarded as being determined

by a potential, the external CO_2 concentration, and a series of resistances, namely, the stomatal resistance to diffusion, the resistances in the intercellular space system and in the liquid diffusion path up to the chloroplast surface, and the "resistances" in the photochemical and chemical phases of photosynthesis. Evidence is presented to show that the observed effect of seasonal changes and of changes in light intensity upon assimilation rate is in part an effect upon the stomatal resistance to diffusion, and in part an effect upon the "resistances" in the photochemical and chemical phases of photosynthesis. There may also be an effect on the resistances in the liquid diffusion phase.

(8) The bearing of these results on the general problem of the relation of assimilation rate to carbon dioxide concentration and to light intensity is briefly discussed, and an attempt is made to formulate a system which takes into consideration the resistances in the diffusion phase as well as the interaction between light and CO_2 in the reactions at the chloroplast surface. It appears that the form of the relation between CO_2 and assimilation, or between light and assimilation, may be profoundly modified by alterations in the relative magnitude of the resistance in the diffusion phase; and it is suggested that the diverse types of inter-relationship exhibited, for example, by the results of Blackman and Smith for Elodea, and of Harder and Warburg for other water plants, may all be particular cases of a more general scheme.

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The North Chinese Species of the Genus Phlebotomus (*Diptera, Psychodidæ*).

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(Communicated by Dr. H. H. Dale, Sec. R.S.—Received January 21, 1928.)

In an earlier paper (1926) we noted the more important differential characters of the adults of the three Chinese species of the Genus *Phlebotomus*, but, as in 1925, we arrived too late in the country to study their early stages, we were unable to record any observations on them. In the present paper we propose to complete our studies of the three species, by describing the differential characters of their early stages, and also we are now able to settle the status of the species with recumbent hairs, which was recorded in the paper noted above as a variety of *P. perturbans* de Meijere. Recently, Prof. de Meijere very kindly sent to one of us (W.S.P.) co-types of his species, and permitted the making of microscopic preparations of the male terminalia and the female pharynx. A study of these preparations has enabled us to settle the identity of the Chinese species allied to *P. perturbans*, and as it proves to be distinct from the Javanese species we propose for it the name *P. taianensis*. Unfortunately we have not been able to settle the identity of the erect-haired species allied to *P. sergenti* Parrot, as it has not been possible to examine and compare the pharynx of a typical female of the latter.

The classification of the species of *Phlebotomus* is at present in a state of flux owing to certain new taxonomic characters recently noted by Adler and Theodor (1926), which promise to be of considerable value in distinguishing the females of this genus.

It is well known that although the males of *Phlebotomus* can be identified by the characters of the terminalia, it is almost impossible to be sure of the identity of the females unless these are either bred or taken *in copula*. In the past, emales have been identified, with difficulty, mainly on the characters of the antennæ and palps, and also by the venation. Sinton (1925) failed to find any reliable taxonomic characters in the structure of the female terminalia. Adler and Theodor (1926), when studying the species of the *Minutus*-group in Palestine, noted that the structure of the mid-pharynx (buccal cavity) varied in the species they examined, as did also that of the spermatheca ; they noted some correlation between the palpal formula and the characters of the mid-pharynx in the females, but in the males, on the other hand, there was no correlation between the characters of these two structures.

Sinton (1927), following up the work of Adler and Theodor, applied these new taxonomic characters in classifying the Indian species of *Phlebotomus*. He notes that in the erect-haired group the characters of the spermatheca are of use in separating the females, as in this group the organ is well chitinized and has a crenulated outline. It varies within wide limits in the species and affords a certain means of separating the females. In the recumbent-haired group, on the other hand, the spermatheca is lightly chitinized, so that its structure affords no reliable characters for separating the females; in this group, however, the structure of the pharynx is of use in identification.

COMPARATIVE NOTES ON THE EARLY STAGES OF THE CHINESE SPECIES OF THE GENUS *Phlebotomus*.

The Egg.

As the general structure of the egg, larva and pupa of *Phlebotomus* have been fully described by Grassi (1907) and Newstead (1911), no useful purpose would be served by redescribing them; consequently, we shall merely note the characters of taxonomic importance in separating the early stages of the three Chinese species known at present.

The egg (fig. 1, **b**) of *P. major chinensis* measures 0·44 mm. in length. The chorion exhibits elongated areas of varying length and width, clearly demarcated from each other by dark lines, as illustrated in fig. 1, **b**. The egg (fig. 1, c) of *P. sergenti* var. measures 0·35 mm. in length and the chorion is divided into small, somewhat hexagonal areas, separated from each other by raised lines which project from the surface of the egg, as depicted in fig. 1, c. The egg of *P. taianensis* n. sp. measures 0·39 mm. in length, is narrower than either of the eggs of the other two species, and the ends are more pointed ; the surface is marked by smaller, regular, quadrilateral areas, as shown in fig. 1, a.

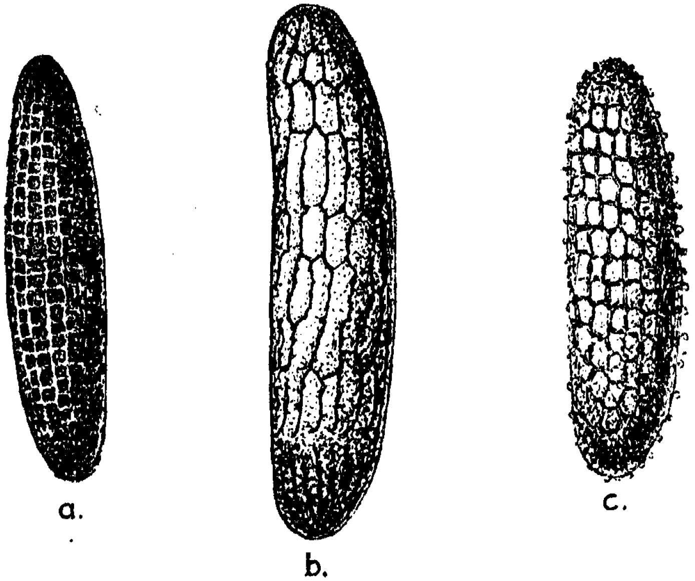


FIG. 1. Eggs of the three Chinese species of *Phlebotomus*. **a**, *P. taianensis* ; **b**, *P. major chinensis* ; **c**, *P. sergenti* var.

First Larval Instar.

The general structure of the larva of *Phlebotomus* is represented in the various figures. The head is well developed, the mouth parts adapted for biting (mandibulate), and eyes are absent. The three thoracic segments are distinct, and there are ten abdominal segments ; the posterior spiracle is located on the eighth and the caudal bristles on the ninth abdominal segment. Ventrally, abdominal segments one to eight inclusive are provided each with a fleshy pseudopod.

The first larval instar of *P. major chinensis* (fig. 2) measures about 0·6 mm. in length. The important taxonomic characters, by which it can be distinguished from the first larval instar of the other two species, are found in the structure and arrangement of the bristles. The two posterior frontal bristles (fig. 2, *p.f.b.*) are long and finely branched to the tip, which ends in a small, clear, globular swelling ; they are situated in front of the egg-breaker, which is always well developed in the first larval instar of *Phlebotomus*. This type of bristle is present on the body of the larva of this species in all its instars, but the globular end is better developed in the body bristles. The pair of anterior frontal bristles (fig. 2, *a.f.b.*) is situated on the front, nearer the middle line just behind the level of the antennæ ; they differ from, the posterior pair as they are not so thickly branched, and end in points. The posterior clypeal bristles (fig. 2, *p.cl.b.*) are long and unbranched, and are situated on each side of the middle line of the most prominent part of the clypeus ; the anterior pair (fig. 2, *a.cl.b.*) is very short, and situated just posterior to the clypeo-labral suture. The vertical bristles, upper and lower, are similar in structure to the posterior frontal bristles. The dorsal genal bristle is long and similar in structure to the frontal bristles ; the remaining genal bristles are simple, as shown in fig. 2.

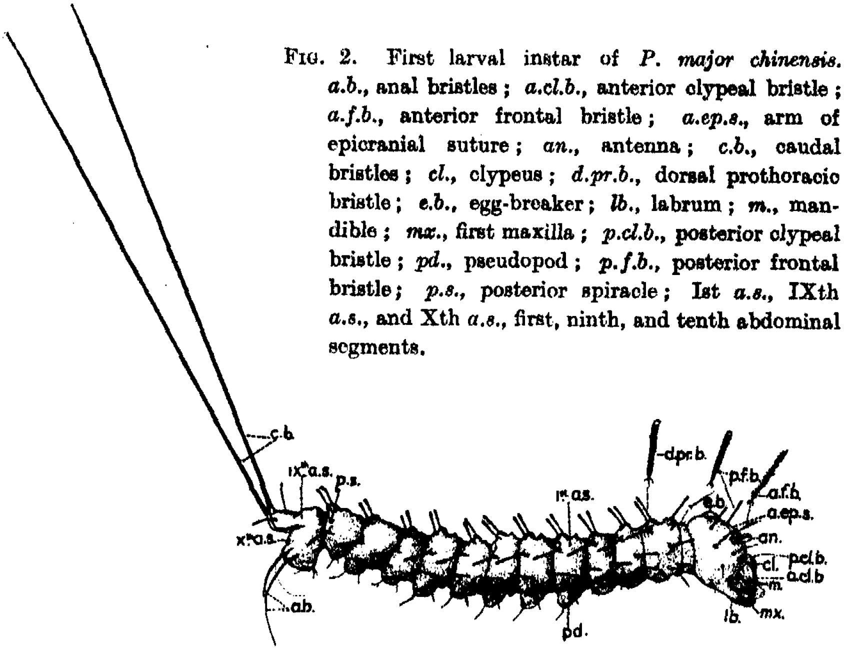
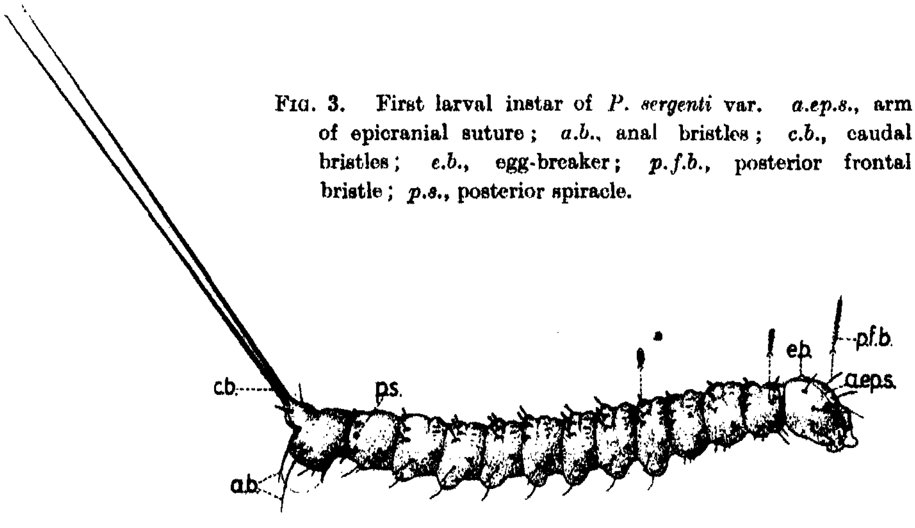


FIG. 2. First larval instar of *P. major chinensis*. *a.b.*, anal bristles ; *a.cl.b.*, anterior clypeal bristle ; *a.f.b.*, anterior frontal bristle ; *a.ep.s.*, arm of epicranial suture ; *an.*, antenna ; *c.b.*, caudal bristles ; *cl.*, clypeus ; *d.pr.b.*, dorsal prothoracic bristle ; *e.b.*, egg-breaker ; *lb.*, labrum ; *m.*, mandible ; *mx.*, first maxilla ; *p.cl.b.*, posterior clypeal bristle ; *pd.*, pseudopod ; *p.f.b.*, posterior frontal bristle ; *p.s.*, posterior spiracle ; Ist *a.s.*, IXth *a.s.*, and Xth *a.s.*, first, ninth, and tenth abdominal segments.

The twenty-two prothoracic bristles are arranged in two bands, an anterior and posterior, and in each there are dorsal, lateral and ventral bristles. The dorsal bristles of the second (outer) pair of the anterior band of the prothorax are short, unbranched and spine-like. The ventral bristles of both bands, as in all the larvæ of the three species, are pointed and not globular at their apices. All the remaining prothoracic bristles are finely branched, and end in small, clear globules. The arrangement of the bristles on the meso- and meta-thorax, and on the abdominal segments is clearly shown in the figure ; except for the length of the dorsal and lateral pairs, these bristles afford no special characters of taxonomic value. The posterior spiracle is situated close to the posterior border of the eighth abdominal segment. The two caudal bristles are strongly developed in all the first stage larvæ of *Phlebotomus*, and vary in length in the different species. In the first stage larva of *P. major chinensis* both bristles are a little longer than the larva itself, and in the egg are folded back. The two large unbranched postero-ventral anal bristles on the tenth abdominal segment are long, and the lower is approximately twice the length of the upper.

The bristles on the head of the first larval instar of *P. sergenti* var. (fig. 3) differ from those of the first larva of *P. major chinensis* in that they are shorter. The frontal and vertical bristles are not thickly branched, and end in points. The other head bristles are shown in the figure. The thoracic and abdominal bristles are in marked contrast with those of the first larva of *P. major chinensis*, as they are all short, especially the dorsal and lateral bristles. The second dorsal bristle of the anterior prothoracic band is small and branched, not spine-like. The dorsal bristles are shortest on the eighth abdominal segment. The two caudal bristles on the ninth segment are a little shorter than the body of the larva.

FIG. 3. First larval instar of *P. sergenti* var. *a.ep.s.*, arm of epicranial suture ; *a.b.*, anal bristles ; *c.b.*, caudal bristles ; *e.b.*, egg-breaker ; *p.f.b.*, posterior frontal bristle ; *p.s.*, posterior spiracle.



The bristles on the head of the first larval instar of *P. taianensis* (fig. 4) are somewhat similar to the corresponding bristles on the first stage larva of *P. major chinensis*. The posterior frontal pair and the upper vertical are finely branched and each bristle ends in a globular swelling. The bristles of the anterior frontal pair are markedly elongated and unbranched; and their structure renders identification of the larva of this species easy. The middle dorsal bristle of the anterior prothoracic band is long and thickly branched at its distal end; the outer dorsal bristle of the anterior prothoracic band is similar to that of the first larva of *P. major chinensis*, but is a little smaller. The remaining dorsal thoracic and abdominal bristles are short and decrease in length from before backwards, those on the eighth abdominal segment being the shortest. The lateral thoracic and abdominal bristles are long. The lower of the two unbranched lateral anal bristles on the tenth abdominal segment is about three times the length of the upper bristle. The caudal bristles are extremely long, and are about one and one-third as long again as the body of the larva. These taxonomic characters are clearly shown in the figure (fig. 4).

Fourth Larval Instar.

The head bristles of this stage of *P. major chinensis* (fig. 5) are a little shorter than those of the first instar, and the posterior, frontal, and upper vertical bristles are pointed at their extremities. In the fourth instar of all three species there are three pairs of dorsal bristles on each of the thoracic and abdominal segments, and the prothorax has a well-

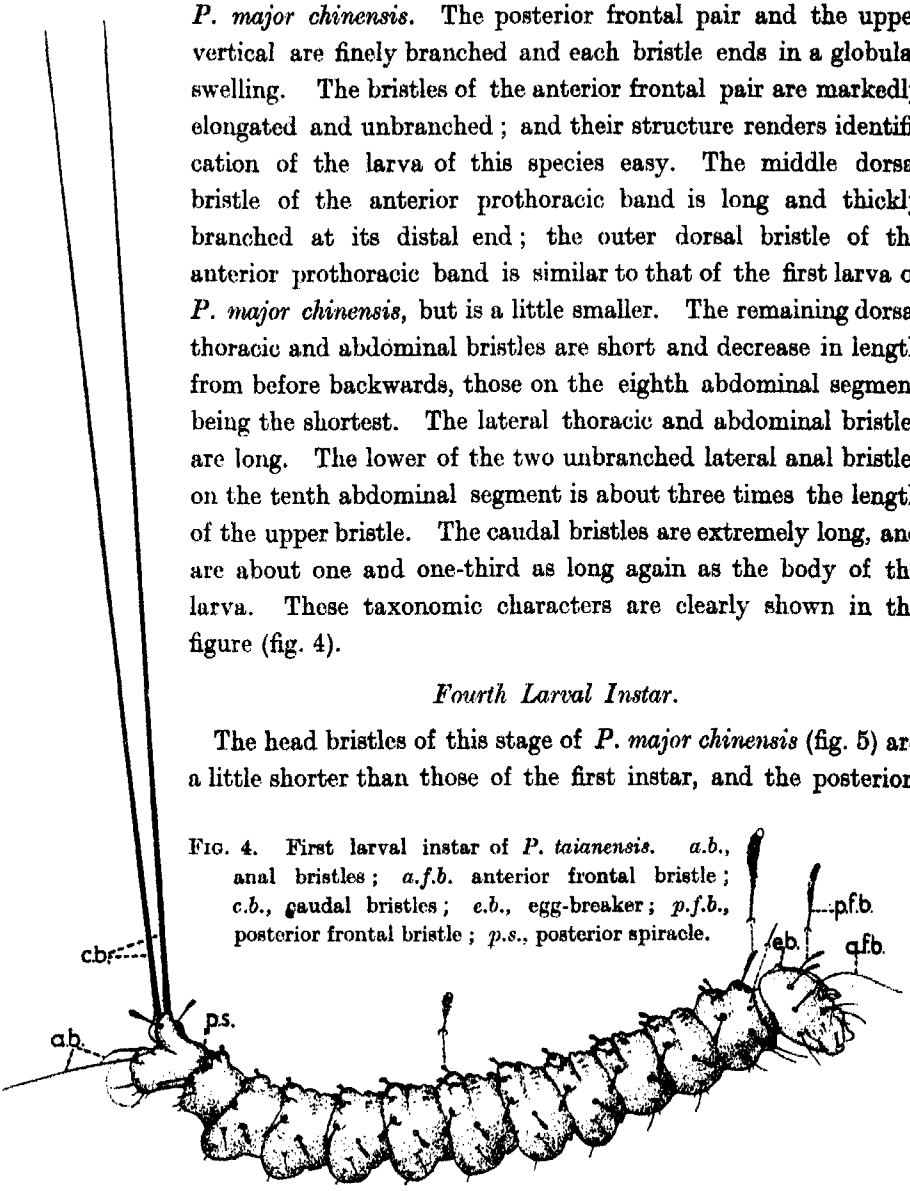


FIG. 4. First larval instar of *P. taianensis*. *a.b.*, anal bristles; *a.f.b.* anterior frontal bristle; *c.b.*, caudal bristles; *e.b.*, egg-breaker; *p.f.b.*, posterior frontal bristle; *p.s.*, posterior spiracle.

developed anterior band. In the fourth larval stage of *P. major chinensis* there is a small, spine-like bristle in the anterior prothoracic band directly in front of the anterior spiracle, and a minute branched bristle between the lower lateral bristles of each band on the prothorax. On the meso- and meta-thorax there is a similar branched bristle between the two lateral bristles. The structure and arrangement of the bristles is illustrated in fig. 5. Four caudal bristles are present in the second, third and fourth larval stages of *Phlebotomus* and are arranged as shown in the drawing; the outer pair is always shorter than the inner, and in the fourth stage larva of *P. major chinensis* the bristles of the inner pair are about two-thirds the length of the larva itself and about one-third as long again as those of the outer pair.

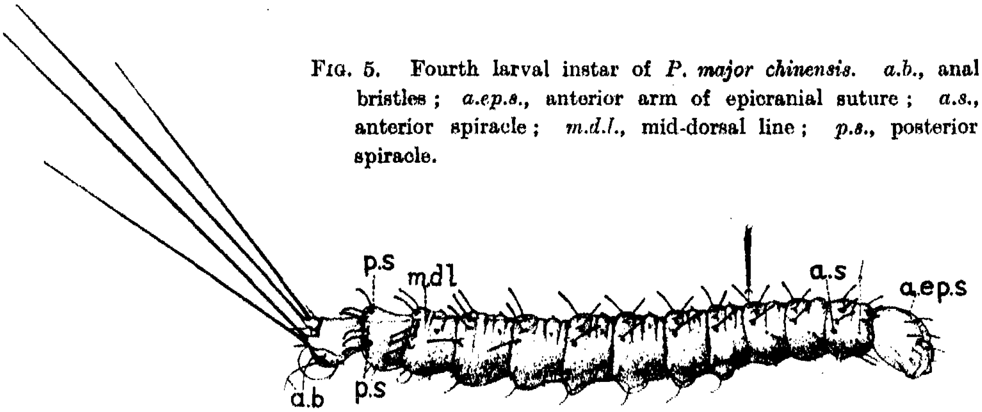


FIG. 5. Fourth larval instar of *P. major chinensis*. *a.b.*, anal bristles; *a.ep.s.*, anterior arm of epicranial suture; *a.s.*, anterior spiracle; *m.d.l.*, mid-dorsal line; *p.s.*, posterior spiracle.

In the fourth stage larva of *P. sergenti* var. (fig. 6) the head bristles are also similar to those of the first larval stage. The small bristle of the anterior prothoracic band directly in front of the anterior spiracle is minutely branched, and not spine-like as in the fourth larval stage of *P. major chinensis*, and this character alone is sufficient to differentiate the two larvæ. In the meso- and

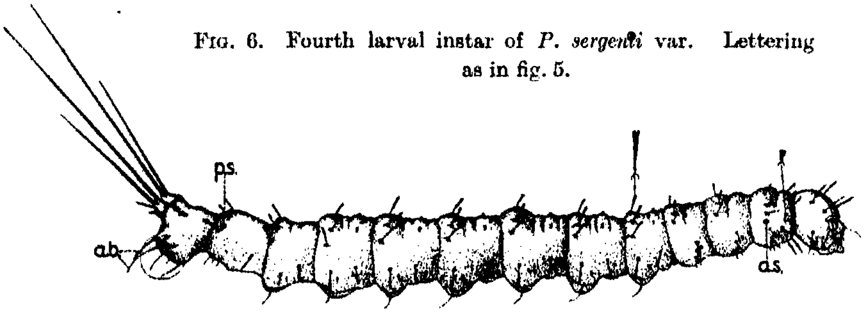


FIG. 6. Fourth larval instar of *P. sergenti* var. Lettering as in fig. 5.

meta-thorax the minute branched bristle is absent above and behind the larger lateral bristle, as shown in fig. 6. The dorsal abdominal bristles are a little more than half the length of the corresponding bristles of the fourth stage larva of *P. major chinensis*. The caudal bristles are comparatively short, the inner pair being about twice the length of the outer.

In the fourth larval instar of *P. taianensis* n.sp. (fig. 7) the head bristles are similar to those of the first instar, but the unbranched anterior frontal bristle, which is so characteristic of this larva, is shorter than the corresponding bristle of the first larva. The middle dorsal bristle of the anterior prothoracic band is only about half the length of the other two, and the small internal bristle is spine-like, as in the corresponding larva of *P. major chinensis*. All the branched thoracic and abdominal bristles are club-shaped, the branches being more numerous towards the distal end, whilst the basal end of the shaft is almost bare. The dorsal abdominal bristles decrease in length from before backwards and are shortest on the eighth abdominal segment. The unbranched, lower lateral anal bristle on the tenth abdominal segment is very long, as in the first larval instar. The caudal bristles are also long and the outer pair is only a little shorter than the inner one.

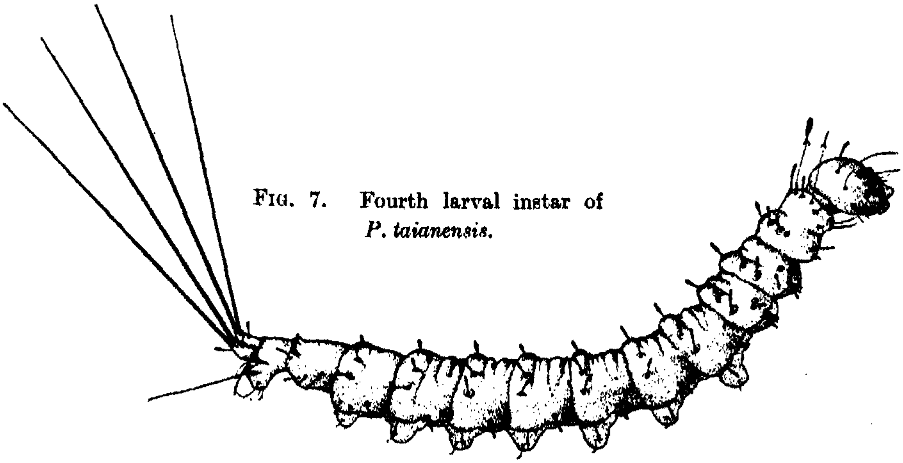


FIG. 7. Fourth larval instar of *P. taianensis*.

The Pupa.

It is well known that in the pupa of *Phlebotomus*, the last larval skin is always attached to the end of the abdomen, and therefore having noted the larval chætotaxy, there should be no difficulty in identifying the pupæ of the three Chinese species. The pupa of *P. major chinensis* has a pair of long, stout unbranched bristles close together on the side of the meso-thorax. The pupa of *P. sergenti* var. has a single small bristle in the same situation. No bristle was found in this situation on the three pupæ of *P. taianensis* examined by us.

Adults.

The main distinguishing characters of the adults have already been noted in our previous paper (1926). Except in the case of rubbed specimens, it is possible after a little practice to identify the females of the two species of the erect-haired group with the aid of a pocket lens; in *P. major chinensis* the thoracic hairs are much darker than those of *P. sergenti* var. As the characters of the wing venation are not very reliable, and are always subject to variation, and as in order to be certain of the relation of the veins to each other, it is necessary to remove the wing and mount it separately, it is better to examine the structure of the spermatheca in any doubtful specimen, since it affords a very reliable means of separating these two species. The fly, preferably a dry specimen, should be placed in weak caustic potash for several hours, and after washing and dehydration, the spermatheca should be dissected out and mounted on a slide in carbol alcohol, ringing the coverslip with Canada balsam.

The spermatheca of *P. major chinensis*, two of which from separate specimens are illustrated in fig. 8 *a* and *b*, is elongated and torpedo-shaped, with a crenulated outline, and the body of the organ seems to be partitioned by dark lines, varying in number from 11 to 13. Its length varies from 98 to 110μ, excluding the tuft of fine hairs at the distal end. The spermatheca of *P. sergenti* var. (fig. 8, *c* and *d*) is very much smaller, and varies in length from 18·5 to 22μ; usually it has three lines apparently dividing it into separate partitions. The

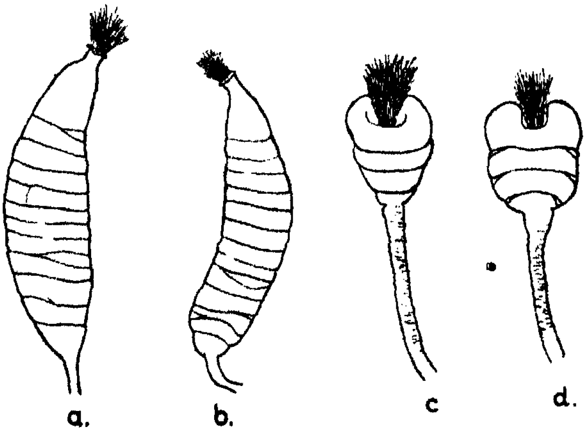


FIG. 8. Spermathecæ of *P. major chinensis* (*a* and *b*), and of *P. sergenti* var. (*c* and *d*).

spermatheca of the recumbent-haired species *P. taianensis* can at once be distinguished from that of either of the other two species, as it is a long sausage-shaped organ without any crenulated margin and also with no dark lines.

The male of *P. major chinensis* can be readily distinguished from *P. sergenti* var. by its darker thoracic hairs, and by the presence in the latter of a brush of hairs on each side at the base of the inner surface of the superior clasper. There are five macrochætæ on the distal segment of the superior clasper of *P. major chinensis*, whereas there are only four on the corresponding segment of *P. sergenti* var.

As already noted above, we have now been able to satisfy ourselves that the small recumbent-haired species is distinct from *P. perturbans* de Meijere. The females can be readily separated by the characters of the mid-pharynx (buccal cavity). In the mid-pharynx of *P. taianensis* the pigmented area on the dorsal wall is shaped like a half moon (fig. 9, *b*), and along its nearly straight posterior border there is a single row of about 56 minute spines. The pigmented area varies in length from 30 to 37μ, and is 75μ wide. The spines measure from 3·7 to 4μ in length. The mid-pharynx measures 92·5μ at its widest point.

The mid-pharynx of *P. perturbans* de Meijere (fig. 9, *a*) is very much smaller measuring 74μ at its widest point in the single specimen examined. The dorsal pigmented area is small and of a much lighter colour than that of *P. taianensis*; it barely reaches to the lateral margins of the mid-pharynx, and measures 18·5μ in length and 37μ in width. The mid-pharynx at this point has a single

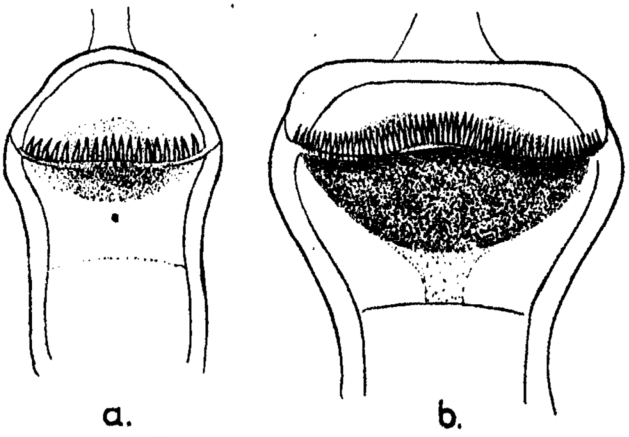


Fig. 9. Mid-pharynx (buccal cavity) of *P. perturbans* de Meijere (a) and of *P. taianensis* (b).

row of 18 rather widely separated stout spines; the longest measures 11μ in length.

The male terminalia of *P. taianensis* are illustrated in fig. 10. The distal segment (*d.sp.c.*) of the superior clasper measures about 0·14 mm. in length, and is armed with four macrochætæ, two situated at the apex, and two close together on a tubercle nearer the distal than the proximal end of the segment. The two distal macrochætæ measure 100μ and 112μ respectively; their structure and relation to each other is shown in the drawing. The other two macrochætæ measure 85μ and 107μ respectively. The proximal segment (*p.sp.c.*) of the superior clasper is broad, and measures 0·29 mm. in length, almost exactly double the length of the distal segment. The inferior clasper (*in.c.*) is long and narrow and is 0·25 mm. in length. It is armed at its apex with about ten long hairs, the calyces of which are shown in the drawing. The submedian lamella, or cercus (*c.*), a paired appendage, is broad and pointed and bears numerous hairs. The intermediate appendage (*i.a.*) is broad basally, and narrows apically, ending in a beak-like point; it measures 24 mm. in length. The paired penis-guards (intromittent organ) are each about 100μ long; the genital filaments are well protruded. The pompetta (*p*) measures 92μ in length.

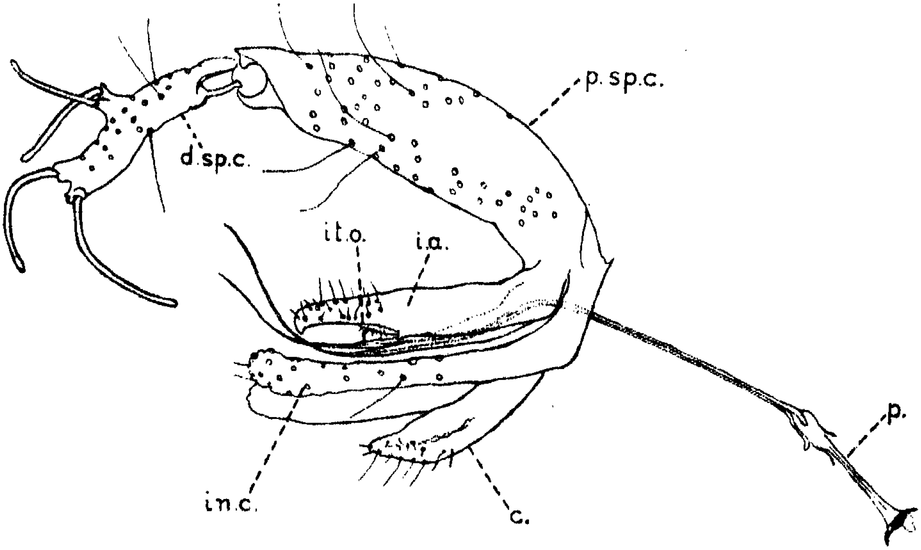


FIG. 10. Terminalia of male *P. taianensis*. *c.*, cerci or submedian lamellæ; *d.sp.c.*, distal segment of superior clasper; *i.a.*, intermediate appendage; *in.c.*, inferior clasper; *it.o.*, intromittent organ; *p.sp. c.*, proximal segment of superior clasper; *p.*, pompetta.

The male terminalia of *P. perturbans* de Meijere are illustrated in fig. 11. The distal segment (*d.sp.c.*) of the superior clasper measures about 0·9 mm. in length, and is armed with four long terminal macrochætæ, two dorsal and two ventral, situated on prominent tubercles.

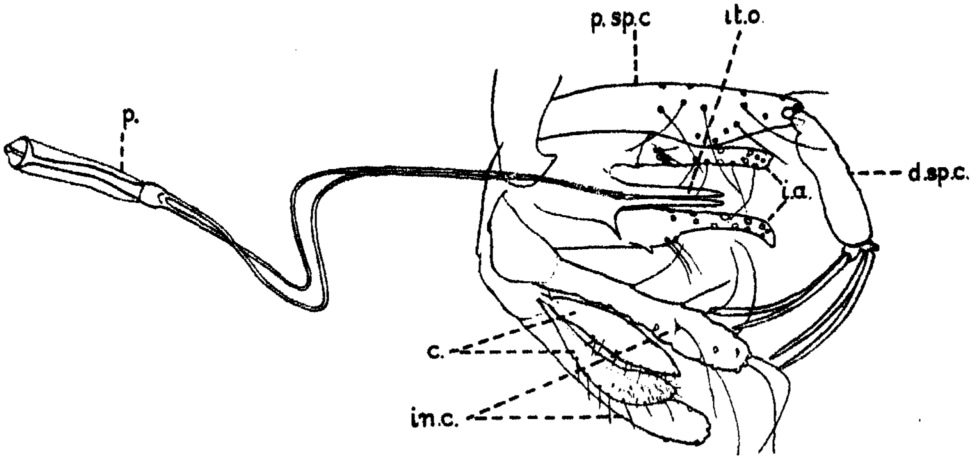


FIG. 11. Terminalia of male *P. taianensis*. Lettering as in fig. 10.

The ventro-external and the dorsal macrochætæ each measure 100μ in length. The ventro-internal macrochæta is spathuliform, and measures 110μ in length. The proximal segment (*p.sp.c.*) of the superior clasper is a little wider than that of *P. taianensis*, and measures 0·24 mm. in length. The inferior clasper (*in.c.*) is longer and wider than that of *P. taianensis* and slightly club-shaped; it measures 0·19 mm. in length and, like that of *P. taianensis*, is armed at its apex with a group of about ten long hairs. The submedian lamella, or cercus (*c*), another paired appendage, is leaf-like, and in side view appears club-shaped. The intermediate appendage (*i.a.*) is long and broad for more than half its length, becoming narrow distally and ending in a broad beak; it measures 0·18 mm. in length. The paired penis-guard (*it.o.*) is about 82μ in length; in the specimen the genital filaments are withdrawn. The pompetta (*p.*) measures 112μ in length. From the above description of the terminalia of the two species it will be noted that the Chinese species, belonging to the recumbent-haired group, is distinct from the Javanese species.

The question naturally occurs as to whether the Chinese species is identical with the Indian species named *P. perturbans* by Annandale. A comparison of the characters of the male terminalia of the Indian species, which Sinton has described in great detail, shows that they are most probably distinct, as will be noted from a comparison of the measurements of the different appendages of the two species given below in tabular form :—

		P. perturbans. (Indian form.)	*P. taianensis.*
Superior clasper	Proximal segment	0·42 mm.	0·29 mm.
	Distal segment ..	0·25 ,,	0·14 ,,
Intermediate appendage		0·25 ,,	0·24 ,,
Intromittent organ		0·14 ,,	0·10 ,,
Inferior clasper		0·33 ,,	0·25 ,,
Apical macrochætæ of superior clasper		90μ	100 & 112μ
Median ,, ,, ,,		60 & 90μ	85 & 107μ.

The shape of the distal segment of the superior clasper, as depicted by Sinton, is markedly different from that of *P. taianensis*, and the two apical macrochætæ are equal in length, while those of the Chinese species are unequal. As far as we are aware, Sinton has not depicted, or described, the characters of the mid-pharynx of the Indian form of *P. perturbans*.

The following is a more complete description of *P. taianensis* than given in our previous paper :—

Phlebotomus taianensis n. sp.

Male.—A medium-sized dark-brown species with dark recumbent hairs on the dorsal surface of the abdomen, and measuring from 2 to 2·2 mm. in length. Palpal formulæ 1, 2, (3, 4), 5 ; segment 5 is long and slender, about equal in length to segments 3 and 4. Antennal formulæ 1/III–XV.

The legs are long, and the second tarsal segment is about half the length of the first. The wing is lancet-shaped, and the posterior border is not more arched than the anterior. Vein R_1 overlaps vein R_2 by about half the length of the latter. Vein R_2 is equal in length to the radial stem between the origins of veins R_2 and R_3, and that of vein R_4. The origin of the latter is much nearer the base of the wing than the origin of the branches of the median.

The male terminalia are not particularly prominent. The proximal segment of the superior clasper is long and very broad, and is about twice as long as the distal segment ; it bears numerous long hairs. The distal segment is narrow and has a marked prominence about two-thirds the distance from its base. It is armed with two long apical macrochætæ unequal in length, and two situated close together on the prominence, also unequal in length ; all the macrochætæ are dilated at their extremities when seen from their concave sides, but appear pointed when seen laterally; one of the distal macrochætæ is distinctly spathuliform. The intermediate appendage is about two-thirds the length of the proximal segment of the superior clasper. The inferior clasper is long and narrow, and is only slightly dilated at its distal extremity; it is about as long as the proximal segment of the superior clasper. The submedian lamella is broad and bluntly pointed. The pompetta measures 92μ in length.

Female.—The female is similar in colour to the male, but, if anything, is darker, the hairs markedly so, and the recumbent hairs on the dorsal surface of the abdomen form a thick layer. The pigmented area on the dorsal wall of the mid-pharynx is half-moon shaped, and along its posterior border is armed with about 56 spines; it varies from 30 to 37μ in length and is 75μ broad. The spines measure from 3·7-4μ in length. Palpal formulæ 1, 2, 3, 4, 5, and segment 5 is longer than segments 2 and 4 together. Antennal formulæ 2/III-XV. Legs similar to those of the male. The wing, on the other hand, is broader in the female. Vein R_1 overlaps vein R_2 by about two-thirds the length of the latter. Vein R_2 is longer than the portion of the radial stem between the origins of the veins R_2 and R_3, and that of vein R_4, and the latter portion is equal to the part of the radial stem between the origin of vein R_4 and the vein R_5. The origin of vein R_4 is nearer the base of the wing than the origin of the branches of the median.

NOTES ON THE BIONOMICS OF CHINESE SPECIES OF *Phlebotomus.*

P. Major chinensis.

In nature, this species normally seems to have only a single brood each year and passes the winter in the larval stage. In 1926 the first specimen was brought to the laboratory at Tsinan on May 22, after which date the numbers gradually increased until about the middle of June, when several hundred were caught each day. Towards the end of the month the numbers began to fall rapidly, and after the middle of July only occasional specimens were obtained, which continued until September 12. The following year at Wei-hsien, a town between Tsinan and the coast, the occurrence of *P. major chinensis* closely agreed with that at Tsinan, the flies first appearing at the end of May and lasting for about six weeks, after which only occasional specimens were seen. In Chihli Province, north of Shantung, the season is one or two weeks later, but all our records agree with the generally expressed Chinese statement that the sandfly is only prevalent during the early summer, and disappears about the time of wheat harvest, when the summer rains result in the formation of pools and streams, that afford plenty of opportunity for the multiplication of the mosquitoes which take their place.

In the laboratory no difficulty was experienced in getting the females to lay eggs in the breeding pots described previously (Patton and Hindle, 1927). As in all stages of *Phlebotomus*, the eggs are rapidly killed by desiccation and have to be kept moist in order to ensure their hatching. Excessive moisture does not seem to affect the early development, for a batch of eggs which was totally submerged for twenty-four hours produced as large a proportion of larvæ as eggs kept under more normal conditions.

At room temperature, 25°–30° C., the larvæ generally emerged after 10–11 days' incubation period. In our experiments the larvæ were usually fed on bat fæces, but some were reared on rabbit dung, and in Peking, Young and Hertig employed hamster fæces. It is probable that any decaying or partially digested organic matter would serve as food. In our experience it was found advantageous to keep a fairly large number of larvæ—at least a hundred—in each pot. When fewer numbers were present, moulds often grew in great abundance over the surface of the food material, with fatal results, whilst in pots containing many individuals the larvæ seemed to be able to keep down the growth of these fungi by feeding on them.

As in other species of *Phlebotomus* there are four larval stages, and under laboratory conditions the duration of the various stages is shown in the following table:—

Egg to first larval instar	10—11 days.
First larval stage	7—8 ,,
Second larval stage	6—7 ,,
Third larval stage	5—6 ,,
Fourth larval stage	7—9 ,,
Pupal stage	7—10 ,,
Total cycle from egg to adult	42—51 days.

In view of the fact that under laboratory conditions the life-cycle may be completed in six or seven weeks, and since the females begin to lay eggs early in June, one might have expected two broods a year, for the summer continues at least until the end of September, July and August being the hottest months. In the laboratory during 1926, adults first emerged about the middle of July, and occasional individuals continued to hatch out until the end of August. All larvæ which had not pupated by the middle of August remained as such and passed the winter in this stage.

Pupæ from these larvæ were first noticed the following February, and adults began to emerge during the latter half of the month and in March. It should be noted, however, that these individuals were kept in an incubator at a temperature of 16°–24° C., consequently the acceleration in the normal rate of development may be attributed to the higher temperature. It is a curious fact that from eggs laid by the same females and kept under apparently identical conditions, some should complete their development in six or seven weeks whilst others take nine months.

We are convinced that under natural conditions the life-cycle is rarely completed in one season. In the laboratory the larvæ are supplied with an unlimited supply of food material, whilst in nature they live in cracks in the ground, in holes, under bricks, etc., and under such circumstances food has to be searched for, and when found the supply is apt to be limited. Consequently, it is reasonable to assume that the length of the larval stages is much longer than under the artificial conditions of our experiments. From the occurrence of the adult flies it is evident that very few individuals complete their whole development during the summer. The flies which appear at the end of May and early in June probably come from larvæ which had reached the fourth larval stage before the onset of the previous winter. The adults appearing later in June may come from larvæ which had only managed to reach the third stage the previous year, and it is just possible that the isolated individuals seen in August and September represent a second brood, which under exceptionally favourable conditions have managed to complete their development in one season.

The larvæ which hibernated in the laboratory in 1926–27 were mostly in the fourth stage, and whenever examined were noted to be active. They were kept at a temperature of 18°–24° C. throughout the winter. On December 6, some of these larvæ were exposed to a temperature of —11° C., and were all dead by the morning. On December 9, some were exposed to a temperature of 0° C. overnight, and next morning were all alive and active. On December 12, other larvæ were placed in the ice chest until the 28th, when it was noted that all were alive ; on January 7, most of them were dead, the last one being found dead on January 15. From these few experiments it is evident that the larva of this species can withstand occasional freezing. Mr. Harkness, Professor of Physics, Shantung Christian University, informed us that at Tsinan the ground did not freeze below a depth of 10 inches during the winter of 1926–27, although it was considered a very cold season. The larva, therefore, would not need to go very deep into cracks and holes, in order to avoid being frozen.

In contrast with the larvæ of *P. sergenti* var., that of *P. major chinensis* is much hardier and more active, and can live under conditions of dryness and excessive moisture that are inimical to the former.

When the larvæ are full grown and ready to pupate they generally come to the surface of the food material, or if the latter is too damp, climb up the wall of the breeding pot until they reach a drier locality as the pupa requires less moisture than the larva. Once the pupa has been formed the adult insect emerges after a comparatively short interval (7–10 days), and there is no evidence to support the view that this sandfly ever passes the winter in the pupal stage.

At the beginning of the season males are always the first to appear and for the first week or so exceed the females in number, but early in June the latter increase until the proportion of the two sexes is not very dissimilar. As a general rule, the number of females brought to the laboratory greatly outnumbered the males, but as we were paying more for the females and as, owing to their blood-sucking propensities, they would be more liable to be caught, it is probable that our records of wild sandflies do not give a fair indication of the natural ratio. In our breeding experiments the males were also the first to appear, suggesting that their development takes a shorter length of time than the female ; the proportion of the two sexes was approximately equal, however, when all the flies had hatched.

The adults of *P. major chinensis* are much more active and quick in their movements than the other two species and also markedly differ in their feeding habits. As a general rule the female seems to take only one feed of blood and digestion proceeds comparatively slowly, traces of the meal still being present after six or seven days, a feature which probably favours the development of flagellates. Although we succeeded in feeding a number of the females of this species a second time, these only comprised a small percentage of the total. The flies readily copulate in the breeding pots and the female lays its eggs in one batch—about fifty in number—on the sixth or seventh day after feeding and then dies. The average duration of life in the males is slightly shorter than in the female.

P. Sergenti var.

In China this species seems to have at least two broods a year, for although it appears simultaneously with *P. major chinensis* about the end of May, it continues without much diminution in numbers until well on into August, and throughout September occasional specimens can be obtained.

The larva is very sluggish and under adverse conditions, such as excessive moisture or dryness, or an overgrowth of mould, it easily succumbs. It seems unable to become adapted to changes in its environment, which is rather surprising in view of the fact that this species is more abundant than the hardier *P. major chinensis*. The first stage larva, in particular, is very sluggish, hardly moving from the place where it emerges from the egg, in contrast with the active corresponding stage of the former species.

The duration of the various developmental stages is approximately the same as that of *P. major chinensis*. Eggs laid in June gave rise to adults seven or eight weeks later, and we assume that the large numbers of adults occurring in nature during August belong to this second brood.

The adults of *P. sergenti* are much easier to keep in captivity and they will readily feed three, four, and occasionally even five times, before laying their eggs. As a rule, the digestion of blood is very rapid, all traces of it usually having disappeared on the third day after a full meal. Occasionally, however, traces of blood may remain in the gut as long as five days after a meal.

On the whole, this species is the commonest sandfly of North China and in many localities occurs in large numbers. On five occasions we observed the male of *P. sergenti in copula* with the female of *P. major chinensis* ; the reverse was never seen. Unfortunately, owing to lack of time, we were not able to follow the result of this abnormal mating. As it was not specially looked for and could only take place in the few hours before the flies were sorted out, it is probable that such crossing is commoner in nature than the above figures would indicate.

P. Taianensis.

This species is common in the temples in and around Taian, where it was first collected, and occurs from the end of May to the middle of September. Although somewhat local, this species seems to have a wider distribution than the other two, as it is not dependent on human beings for its food supply. At Tsingtao, with the exception of isolated examples of *P. major chinensis*, which all came from one house, this was the only species of sandfly to be found.

In the laboratory the duration of the life-cycle is similar to that of the other two species, and like *P. sergenti* it seems to have at least two broods each year. The larva is much more hardy and active than the other two and can withstand desiccation for a considerable period. Pupal cases of this species were found in November, 1926, by Dr. Hertig and one of the writers (E.H.), at Wo-Fu-Ssu, a temple on the Western Hills about eighteen miles from Peking.

They were in loose earth, attached to small pieces of tiles, at a depth of 6–8 inches below the surface.

P. taianensis seems to feed normally on batrachian or reptilian blood and readily feeds on frogs, toads, lizards and snakes. We have also succeeded in feeding a few individuals on a fowl and on a hamster, so it is possible that it may sometimes feed on man, although we were never able to get any specimens to feed on ourselves. When feeding, this sandfly ingests so much blood that its abdomen becomes almost spherical and afterwards it is very sluggish. The process of digestion is even more rapid than in *P. sergenti*, and it is possible to get them to feed every day or two.

In concluding these notes on the North Chinese species of *Phlebotomus*, we should like to point out again that we have been unable to obtain any evidence of their existence from the Yangtse River Valley southwards, and the present distribution agrees with what is known about the incidence of Kala Azar in China, which also seems to be restricted to the Provinces north of the Yangtse.

We should like to express our heartiest thanks to Mrs. Patton, to whom we are indebted for the drawings illustrating this paper.

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OBITUARY NOTICES

OF

FELLOWS DECEASED.

CONTENTS.

PAGE

Joseph Jackson Lister (with portrait) i

Willem Einthoven (with portrait) v

William Boog Leishman (with portrait) ix

Ernest Henry Starling (with portrait) xvii

J. J. Hilton.

JOSEPH JACKSON LISTER—1857-1927.

THE father and the grandfather of Joseph Jackson Lister were distinguished men of science and Lord Lister, the President of the Royal Society, was his uncle. With a rich inheritance of scientific talent there were combined in his character a strong love of Nature and the industry and patience to combat the handicap of a delicate constitution. He was known to his friends and colleagues as a learned and accomplished naturalist, and he acquired a world-wide reputation for his investigations on the morphology of the Foraminifera.

He was born at Leytonstone in 1857, and in his early years he and his sister used to ramble in Epping Forest guided and stimulated by their father to observe and study the animals and plants of the countryside. He soon became an ardent collector of butterflies and moths and, later on, of beetles to add to the family collections.

His father, Mr. Arthur Lister, was not only a naturalist in the ordinary sense of the term, but had inherited from his father, who had made important improvements in the compound microscope, a love for the study of minute organisms, and, acquiring an interest in the Mycetozoa, had pursued investigations on their structure and life-history which gained for him the Fellowship of the Royal Society. His son consequently was brought up in an atmosphere in which the ordinary pursuit of Natural history was supplemented by a deeper study of the problems of organic life.

Lister was sent to a school at Brighton at the age of nine, but three years later he entered the famous school of the Society of Friends at Oliver's Mount, Scarborough. The headmaster, Mr. Thomas Walton, encouraged the study of natural history, and in expeditions into the country and on the sea shore Lister added rapidly to his knowledge of wild life and to his powers of keen observation. He was not, however, only a naturalist for he took a deep interest in literature and used to enjoy reading Shakspere, Tennyson, Clough and other poets, while the writings of Carlyle were his special favourites, and from them no doubt he gained the interest in intellectual and social problems which was maintained in later years. In this respect too he was probably strongly influenced by an older relation by marriage, Mr. Joshua Rowntree, whom, in his later school days, he used to help on Sundays in an adult school for working men at Scarborough.

Lister entered St. John's College, Cambridge, in 1876, and was one of the small group of students who were fortunate enough to attend the classes of that most inspiring teacher Francis M. Balfour, at that time engaged on his wonderful investigations on the Embryology of the Chick and the Dogfish, which caused such a sensation in the scientific world. Always fond of games and naturally skilful in their pursuit Lister took to rowing as his form of exercise and relaxation at Cambridge, and in 1878 he was made stroke of the Lady Margaret boat and held that position from that time until he went to London in 1882. He led his crew to many victories both on the Cam and at Henley, and was said by a contemporary to have been the best stroke Lady Margaret had ever had.

After taking his degree in the Natural Sciences Tripos he was appointed by Prof. Newton his demonstrator in Comparative Anatomy and gave a course of lectures on the anatomy of the Vertebrata. As one of the four students who attended that course, the writer can say that it was with great regret we learned that he had decided to resign his post in order to study medicine. He then entered University College, London, as a medical student, gained the Liston gold medal in surgery and took the M.R.C.S. qualification. He was not, however, destined to practise in the profession, as his health began to fail and he was strongly urged by his uncle, Lord Lister, to spend some time in travel. He went to the West Indies with several others of his family, and on his return in the autumn of 1887 started again in a sailing ship, the *Paramatta,* on a voyage to Australia and back.

In 1887–1888 he was acting as naturalist on board H.M. Surveying Ship "Egeria," and after going to Christmas Island, Mauritius and the Seychelles, he joined the ship again in 1889 and visited Australia, New Zealand, and several islands in the Pacific. To a man of Lister's wide interests and sterling abilities these wander-years were times not only of intellectual delight but of the steady accumulation of knowledge and ideas by the direct study of Nature in her various aspects. His mind was always alert to notice things bearing on scientific theories in zoology, botany, anthropology, and geology, and he wrote several papers, to which reference will be made later, bearing on observations made during these journeys. He made his collections of animals and plants wisely and efficiently, as much for his colleagues working in the laboratories at home as to satisfy his own personal interests.

A fellow-passenger on board the *Paramatta* writes of her pleasant memories not only of the charm of his companionship during the voyage, but of his kindliness to her children, and of the interest he created by showing them through the microscope, the phosphorescent and other organisms of the ocean.

Returning to Cambridge in 1891, with his health restored, he again accepted appointments in connection with the school of zoology in the University, and also began the series of investigations on the morphology of the Foraminifera which formed the subject of his address when he was made President of Section D at the York Meeting of the British Association in 1906. In 1899 he was made a Fellow of St. John's College, and in 1900 a Fellow of the Royal Society, and for many years he was occupied with the many intellectual activities of University life in Cambridge. In various ways he improved the practical work of the school of zoology there, bringing to bear upon it his exceptionally wide knowledge and experience of living animals, both terrestrial and marine, in their natural surroundings, and his lectures were always remarkably interesting and stimulating. He continued his researches on Foraminifera and Mycetozoa, he gave valuable assistance to Adam Sedgwick in preparing the third volume of his Text-book of Zoology, in which he wrote the articles on the Crustacea and Xiphosura, and he also contributed the articles on Foraminifera and Mycetozoa in Sir Ray Lankester's Treatise on Zoology.

In 1911 he was happily married to Miss Dorothea Marryat, daughter of the late George Selwyn Marryat, and they settled down in a beautiful house in Grantchester. Some years later his health again showed signs of failure, and his visits to the zoological department became less frequent, but, turning again to the interests of his early life, he carried on at home a series of observations on the variations of butterflies and moths, though he has unfortunately left no published papers on this subject. He died in his Grantchester home on February 5, 1927.

Of Lister's published contributions to science it may be said that, although not extensive, they are valuable and interesting records of careful and skilful work done. He could never bring himself to print anything that had not been thoroughly investigated and conscientiously considered. He wrote to express the truth that he had found, and it could always be trusted, and what he has written has stood, and will stand, the test of time. His first paper was written in conjunction with Mr. J. J. Fletcher, on the female organs of reproduction of the Kangaroos, the result of some dissections he had made at the Zoological Gardens in London during the long vacation of 1881.*

In 1888† he wrote an admirable article on the Natural History of Christmas Island, the fauna and flora of which were threatened with destruction in consequence of the operations of a guano company, and in the same year he described the stalked form of the coral Fungia first discovered by Rumphius in the 17th century, and briefly described by Stutchbury in 1830.‡

Arising out of his travels in the Pacific Ocean, he wrote an account of Falcon Island,§ and of the Tonga group‖ in general, in which he made important contributions to the controversy on the origin of coral reefs and to the geology of these oceanic islands. An interesting paper on the natives of Fakaofu (Bowditch Island)¶ was his only contribution to our knowledge of anthropology, but it showed that he possessed a wise insight into the problems of that science.

As a naturalist from his boyhood, he was, of course, greatly interested in the birds of these tropical islands, and his visit to the bird colonies in the Phœnix Islands must have been one of the most thrilling incidents of his life. He published an account of the birds of this locality, including an interesting description of the throat pouch of the male Frigate bird.*

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* 'Proc. Zool. Soc.,' 1881.

† 'Proc. Zool. Soc.,' 1888.

‡ 'Quart. Journ. Micro. Sci.,' vol. xxix.

§ 'Proc. R. Geog. Soc.,' 1890.

‖ 'Quart. Journ. Geol. Soc.,' 1891.

¶ 'Journ. Anthrop. Instit.,' vol. xxi.

On this voyage to Australia in the *Paramatta* he spent much of his time in studying the plankton, and discovered a most interesting and hitherto unknown Metanauplius larva which he assigned, probably correctly, to the group of the Stomatopoda.†

His most important work, however, was the memoir published in the 'Philosophical Transactions' of the Royal Society in 1894 on the life-history of the Foraminifera. He described the megalo- and microspheric forms of the common *Polystomella crispa* of our shores, and showed that the microspheric form gives rise to a brood of megalospheric young, and that the megalospheric form gives rise to an immense number of minute flagellate zoospores of uniform size which in their turn give rise to the microspheric forms. He proved in this way that the two forms were not respectively male and female, as was sometimes assumed, but two distinct generations, which, in Polystomella at least, appeared to alternate. The flagellate zoospores produced by the megalospheric form might be facultative conjugants, and therefore sexual spores, but this he failed to prove. This memoir was illustrated by several excellent drawings of the living organisms, a testimony to his accuracy of observation and his delicate skill as a draughtsman.

Other papers dealing with the phenomena of dimorphism in Orbitolites, Triloculina, Biloculina, and the Nummulites were published in the 'Cambridge Philosophical Transactions,' but a great deal of valuable new work on the Foraminifera was also included in the article he wrote on the group for Lankester's 'Treatise on Zoology.' These researches brought him a great reputation, and, as Prof. F. E. Schulze, of Berlin, wrote, "won for him wide recognition among zoologists of all countries, but especially among his fellow-workers in that field of study."

Reference may also be made to a paper he wrote in 1900 on '*Astrosclera willeyana*.'‡ He gave this name to a hard calcareous structure found by Dr. Willey on a coral reef in the South Seas, the nature of which was the subject of much speculation and of some dispute in the Laboratory. By skilled manipulation Lister settled the question through showing that the structure contained the characteristic collar-cells of a sponge.

When the Great War came upon us and zoological investigation had, to a great extent, to be set aside, Lister turned his great manual dexterity to the making of artificial limbs for wounded soldiers. He was a man who, inheriting

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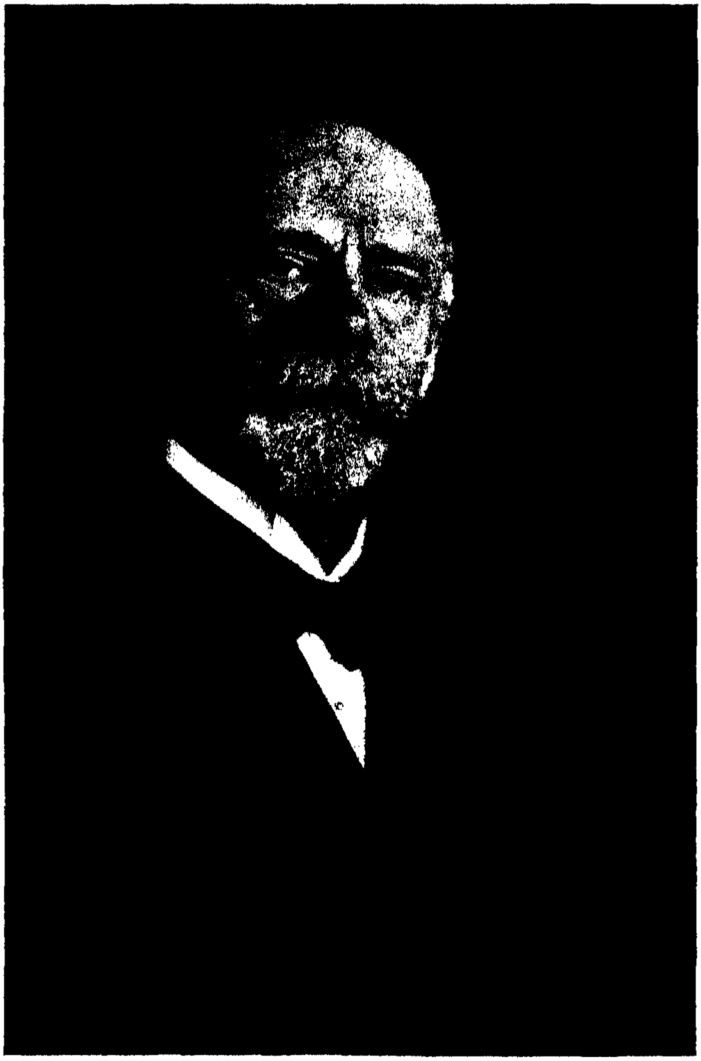
* 'Proc. Zool. Soc.,' 1891.

† 'Quart. Journ. Micro. Sci.,' 1898.

‡ Willey's 'Zoological Results,' Part IV.



W. Einthoven

as he did the kindliness and sympathy of his family, and having been influenced in his early life by the principles of the Society of Friends to which they belonged, must have felt keenly the horrors of war, and he did all in his power to help the sufferers.

Lister had a large number of loyal friends who held him in high regard, and there are many younger men who, having passed through their three years' course in Cambridge and gone out into the world, are grateful to him for his ever-ready help from the stores of his wide knowledge and experience, and for his sympathy in their difficulties and disappointments. S. J. H.

WILLEM EINTHOVEN—1860-1927.

WILLEM EINTHOVEN, whose death was announced on 29th September, was born in May, 1860, at Samarang in Java. He was the son of a doctor practising in that place, and was taken to Holland by his mother with five other children when his father died in 1870. They settled at Utrecht, and here Einthoven entered the University as a student of medicine in 1878, to study physics under Ballot and physiology under Donders. His first research was undertaken with the anatomist Koster and concerned the mechanism of the elbow joint; he became assistant to the ophthalmologist Snellen, and was given his doctorate of medicine at 25 years for an Inaugural Dissertation entitled "Über Stereoskopie durch Farbendifferenz." In the same year he was called to the Chair of Physiology at Leyden, and there he remained, actively engaged in work, until his death in his 67th year.

Early in his career he became interested in instruments recording changes of electrical current or potential, and, perceiving their great importance to physiological research, he deliberately set out to construct an instrument of unsurpassed quickness and sensitivity. The history of the construction of his famous string galvanometer has been written by Einthoven himself in one of his papers. His researches started on the basis of the Deprez-d'Arsonval galvanometer. He soon discovered that the less numerous were the windings of the moving coil of this galvanometer, the greater was its sensitivity, and that the highest sensitivity was obtained when a single winding of very thin wire was employed. It was then (1897) that he became aware of Ader's instrument for recording submarine signals, a galvanometer consisting of a long fibre lying vertically between the poles of a magnet. He continued to enquire into the factors required to produce speed of movement and sensitivity and, by greatly increasing the strength of the magnetic field, by using relatively short and extremely fine conducting fibres, and by employing an optical system of projection of high magnification, eventually constructed a galvanometer greatly surpassing, in continued quickness of response and sensitivity, any that had previously been made.

His first account of this instrument was published in 1903; the sensitivity of the galvanometer, described fully by him in 1909, was already 100,000 times greater than that of Ader's instrument. Although his instrument, regarded as a potentiometer, then stood possessed of little more sensitivity than the best models of capillary electrometer, it surpassed the latter and all previous galvanometers enormously in the quickness of its response. The capillary electrometer had the defects of slow response and overswing; a true curve of potential differences could be obtained only by a tedious analysis of the electrometer curves, an analysis involving considerable possibilities of error. The response of Einthoven's string galvanometer was so quick that very rapid current changes were directly and accurately recorded, no analysis being required subsequently.

He expended a great deal of time and patience on the perfection not only of the galvanometer itself, but of the subsidiary apparatus, particularly plate cameras and rotating time-markers, with a view to reducing to their narrowest limits errors of measurement arising from such sources. He succeeded in reducing errors of time measurement in physiological electrical curves to such minute intervals as 1/10,000 or 1/100,000 of a second; and produced those records of current change upon a photographic network of vertical and horizontal lines, accurately representing time and current magnitude, which have commanded universal admiration for their technical perfection alone.

His galvanometer became the basis of the well-known commercial models of Edelmann, and of the Cambridge and Paul Instrument Company, and of many other patterns. Very many hundreds of these instruments are now in use in physical, physiological and pathological laboratories, and in the hospitals of many countries. Modifications, which have been introduced, other than those of the inventor himself, have consisted almost exclusively of minor changes, adapting the machine for special purposes; in principle it has remained unaltered; in sensitiveness it has greatly increased, but Einthoven's own models have continued until the last to outstrip by far all similar instruments in this respect.

Einthoven was not content merely to place his instrument at the disposal of laboratory workers; he and his collaborators clearly pointed to the many ways in which it might usefully be employed. Following up Waller's demonstration that curves of the heart beat can be obtained by leading off from the limbs of an animal, he laid the basis of human and experimental electrocardiography as these are practised to-day. Modern electrocardiography is the direct outcome of two papers published by him in 1907 and 1908. The leads from the human subject then adopted by Einthoven, and the standardisation of curves then employed by him, have since become universal. The same papers also contained curves of a variety of irregularities of the disordered or diseased heart, and their publication led at once to a complete investigation of cardiac irregularities by this method; the outcome of these researches has been a complete and final analysis of all those irregularities that the human heart commonly displays. This work has confirmed and has widely extended the previous analyses by Mackenzie's method of studying the jugular pulse.

In 1913 he published a fundamental account, enabling the electrical axis of the human heart to be calculated at any phase of the heart's cycle; this, by comparing the curves taken from three separate leads along the sides of an approximately equilateral triangle. This method he reduced to greater exactitude three years later, when he invented a method of recording the three leads simultaneously and accurately. The methods of these papers have been exploited extensively and fruitfully in the study of the normal heart beat and in the elucidation of the disorders of the human auricles termed flutter and fibrillation.

In other papers Einthoven showed how eminently his instrument is suited to the study of electrophysiology generally. Thus, in 1908, in illustrating its application to nerves, he showed that natural afferent impulses, conveyed by the vagus nerve from heart and lungs to brain, can be recorded; more recently (1923) he recorded efferent impulses in the cervical sympathetic and other nerves. His methods have been extensively adopted, largely at his instigation, to study the electromotive changes in somatic nerve and muscle and in the sense organs and secretory glands.

Another and considerable activity of this pioneer was his attempt to record the sounds of the heart beat. For this purpose he used the capillary electrometer as early as 1894, in conjunction with Geluk. Later he devised a microphonic circuit suitable for attachment to his own galvanometer, wherewith sounds ranging to a vibration frequency of 200 per second might be recorded accurately. He showed this device to be suitable for registering the sounds of the human heart, and upon these he made many original observations; it has since been used extensively to study abnormal heart sounds in patients. The heart-sound method devised by him has proved the most accurate we possess in timing the chief mechanical events in the human heart cycle, and has been much employed for this purpose. Its use has been limited, as has to a greater extent that of other devices, by inability of the instrument to follow accurately sound vibrations of very high frequency. Discovering methods of drawing out fibres to an extraordinary degree of thinness, Einthoven in his last years overcame this difficulty; his threads of quartz, two or three millionths of an inch in diameter, would respond directly to sound vibrations of a frequency of 150,000 per second; these movements of a fibre, agitated by sound waves in the air in which it lay, Einthoven recorded photographically.

In these same, and last, years he worked to adapt his string galvanometer as a recorder for wireless waves; he succeeded in directly registering waves transmitted to Holland from the Dutch East Indies.

Einthoven's renown grew steadily, and in the last years of his life many honours were conferred upon him; these culminated in 1924 in the award of the Nobel prize for Medicine, and, in this country, in his election in 1926 to the Foreign Membership of the Royal Society. Honours, however, were to him a smaller recompense than was the knowledge of the benefits which his long and arduous work had conferred upon his fellow-men. To few scientists, perhaps to no physiologist, has the applied value of their discoveries been so abundantly demonstrated as it was to Einthoven in his lifetime; the strength of this demonstration surprised him and gave him deep satisfaction.

Einthoven's work will be remembered for all time for the greatness of its contribution to method. He himself will be remembered by those who knew him personally for his fascinating personality. A man of simple, almost humble, habits, he was untiring in his devotion to work, to the exposition of his views and to the study of related problems. He awakened in both friends and associates a profound admiration, by his genius, by the charming simplicity and directness of his character, by his unusual modesty of thought and manner, by his patience, by his natural and unfailing courtesy, and by his unswerving devotion to truth in the most exacting sense. These noble qualities endeared him to all who knew him well.

T. L.

W.B. Leishman.

WILLIAM BOOG LEISHMAN—1865–1926.

WILLIAM LEISHMAN died on June 2, 1926. He was one of the small band of investigators in our Army Medical Services who, within the last 30 years, have added much to scientific knowledge and incidentally to the prestige of the Royal Army Medical Corps and Indian Medical Service. Amongst this band Leishman was distinguished, not merely for his own researches, but for his untiring and unselfish efforts to further the interests generally of medical discovery in this country.

He had a natural aptitude for scientific inquiry and found pleasure in the pursuit of knowledge and a keen interest in its practical application, but the medical service of the army was always his foremost consideration. The reputation of his corps was indeed very dear to him, and his ambition was achieved when in 1923 he became the Director of the Army Medical Service.

Leishman was born in 1865. His father, Dr. William Leishman, was Professor of Midwifery in the University of Glasgow. After a few years at Westminster School, years to which he always looked back with pleasure, Leishman started on his medical studies at Glasgow and here he graduated in 1886. Entering the army at the early age of 21, he was gazetted a Surgeon in the Medical Staff in 1887, and soon proceeded to India, where he saw active service in the Waziristan Expedition in 1894-95, receiving the frontier medal and clasp. It was characteristic of the man that, at a time when any departure from the ordinary routine of clinical work was rare, he took a microscope with him on his Indian tour.

Returning to England in 1897, he was posted as a Medical Officer to Netley Hospital, then also the Headquarters of the Army Medical School. Almroth Wright was the professor of pathology to the school, and Leishman was attracted to laboratory work under Wright's inspiration, and decided to become a serious student of pathology, if this were possible. It proved possible, and in 1900 he succeeded Major (now Sir David) Semple as Assistant Professor of Pathology at Netley.

On the removal of the Army Medical School to London and on the appointment of Sir Almroth Wright as pathologist to St. Mary's Hospital, in 1903, Leishman became Professor of Pathology at the Royal Army Medical College. For a while he was largely occupied in the organisation of the teaching of his department and his classes became an important element in the training of medical officers, both in their preliminary work on entering the army and in the "promotion courses" then made obligatory for the first time. Leishman took great pains with his teaching work and his courses of instruction were greatly appreciated. At the same time he found opportunities to do some excellent research himself and he inspired a number of young officers to undertake investigations and trained them in scientific methods.

In 1905 he was made a Brevet-Lieutenant-Colonel in recognition of his scientific investigations, and in 1910 he was elected a Fellow of the Royal Society. In 1913 he quitted his chair of pathology at the R.A.M. College to assume the duties of Expert in Tropical Diseases to the Army Medical Advisory Board at the War Office, and in the same year he became one of the original members of the Medical Research Committee.

On the outbreak of the European War, after organising the arrangements for the antityphoid inoculation of the troops proceeding overseas, he quitted the War Office for France, and in October, 1914, was made Adviser in Pathology on the Staff of the Director-General of Medical Services of the Expeditionary Force. Thrice mentioned in Despatches, he received the C.B. (Military), the Legion of Honour (3rd Class) and the American Distinguished Service Medal.

In 1918 he was recalled to the War Office and, on the formation of the new Directorates of Hygiene and Pathology, was made Director of Pathology, and in the same year he was created a K.C.M.G. In 1918, too, he was promoted to the rank of Major-General. In 1923, on the retirement of Sir John Goodwin, Leishman became Director-General, and was promoted a Lieutenant-General, created a K.C.B. and advanced by the French Government to the grade of Grand Officer of the Legion of Honour. He was also a Knight of Grace of the Order of St. John of Jerusalem.

These details will suffice to show that Leishman's accomplishments and public services were universally recognised. Let us turn now to his scientific work. In 1897, when Leishman returned from his tour of foreign service to Netley, the development of bacteriology and protozoology was proceeding apace, and the application of the new knowledge to the problems of tropical medicine was manifest. The microscope had become as important an aid to the diagnosis of tropical diseases as the clinical thermometer. His first original contribution to science, made shortly after his appointment as Assistant Professor under Wright, was an improvement in the method of staining malarial parasites so that they might be more easily detected in a film of blood. Maurer had advanced upon the methylene blue-eosin method of Romanowski by using Nocht's methylene blue ripened by alkali, but his method still involved the use of two solutions, required preliminary fixation of the film and was rather troublesome to manage.

It occurred to Leishman that by precipitating ripened methylene blue with eosin, as Jenner had done with ordinary methylene blue, a double staining compound might similarly be produced which, if also soluble in methyl alcohol, would possess the advantages of Maurer's two solutions and the conveniences of Jenner's single one. Such proved to be the case, and the useful compound stain Leishman had made came into general use for the rapid staining of microscopic preparations, of malarial parasites and other protozoa. Leishman's name became notorious all over the world amongst histologists and tropical practitioners; the chemical structure of the doubly staining compound being obscure, the name of its inventor naturally came to be attached to it.

Leishman's second piece of individual work also involved a development in scientific technique, indicating that he was capable of forging his own tools. At this time, 1900–1901, Wright had launched his experimental trial of anti-typhoid inoculation during the Boer War and was exploiting similar methods in the treatment of staphylococcic infections. This led to an investigation of the physiological mechanisms whereby the resistance occasioned by a previous injection of a killed culture of different microbes was brought about. Whereas inoculation of killed typhoid or cholera bacilli enhanced the bactericidal power of the serum, Wright found that this did not occur after the injection of staphylococci. These organisms, indeed, could be propagated in the serum of either normal or immunised animals and resistance to infection could not, therefore, be attributed to the unaided action of the serum. Wright and Leishman observed, as others had done, that in the blood of artificially immunised animals the microbes were more readily ingested by the leucocytes, but the lack of some method for estimating phagocytic activity prevented quantitative comparison.

Leishman supplied this want. His method was but roughly quantitative, but it was an important step in advance. By its use he was able to arrive at a numerical comparison of the phagocytic activity of the blood of different persons before and after they had been inoculated with killed cultures of staphylococci and *Micrococcus melitensis*, using the average number of microbes ingested by the leucocytes in the blood of a normal person as a standard of reference. He also made the interesting observation that in the case of a patient suffering from intermittent attacks of boils, a fall in this phagocytic ratio synchronised with the attack, whereas during the period of freedom from boils it was above that of normal people.

Leishman did not ascertain whether the increased phagocytic power which he observed was due to an inherent alteration in the leucocytes or to some modification in the serum which favoured the ingestion of the microbes by the phagocytes by its action upon the one or the other. He rested content with the older interpretation of Metchnikoff, that the serum was modified and stimulated the leucocytes to greater efforts, although Denys and Leclef's work had negatived this interpretation in the case of streptococci. The complete analysis of the phenomenon was subsequently made by Wright and Douglas, whose improvements in Leishman's technique permitted them to discover that it was the serum which was modified, and that microbes bathed in such modified serum were altered in such a way that they were subsequently much more readily englobed. This variable property they appropriately designated the "opsonic power" of the serum.

In their earlier experiments Wright and Douglas studied the opsonic power of the serum only of normal uninoculated persons and found that such serum lost its opsonic power after heating to 60° C. Leishman, however, discovered that the serum of individuals convalescent from Malta fever and from men who had undergone antityphoid inoculation retained its power to enhance the phagocytosis of the corresponding microbes after heating to 60°. About the same time, Neufeld and Rimpau discovered a similar thermostable opsonin in the sera of animals immunised against staphylococci and streptococci.

Leishman, therefore, opined that this thermostable property of the serum after inoculation was due to a protective substance other than the opsonins discovered by Wright and Douglas. If it was a different substance it might act in a different way; not upon the microbe but by stimulating the leucocytes themselves to greater efforts, the view originally promulgated by Metchnikoff. In 1905 Leishman published some observations designed to test this. They were not very convincing, as he was careful to point out, but they inclined him still more towards the view of the French school. He suggested the name "stimulins" for the thermostable substances in immune sera, and was rather persistent in his view that they operated upon the leucocytes, notwithstanding that evidence soon accumulated to show that the original observations of Denys and Markl had general application, and that whatever the relationship between these bodies in normal and in immune serum might be, they both increased the phagocytic power of the blood by increasing the palatability of the microbes.

It was at Netley, in 1901, that Leishman made an observation which subsequently proved of import to tropical medicine. In the course of an autopsy upon a soldier who had died of kala-azar, or "dum-dum fever," as it was then called, he examined some films of material from the spleen which he had stained by his method for malaria parasites. The field of the microscope was strewn with small oval bodies, 2-3 μ in diameter and each containing two masses of chromatin staining material, one circular and the other rod-shaped. The appearance was one he had never met with or seen described before. He did not record the observation at the time, pending opportunities for examining material from further cases of the disease, but he made careful notes and drawings of the appearance presented. Two years later, when examining specimens from rats which had died some hours previously from infection with *Trypanosoma Brucei*, he noticed degenerated trypanosomes, which so forcibly reminded him of his earlier observations that they now seemed worthy of publication. Human trypanosomiasis had been recently discovered by Dutton and Forde, so Leishman hazarded the opinion that kala-azar was a disease of similar causation, and suggested that a search for the parasites should be made in cases of the disease in India during life.

Shortly afterwards, Donovan and others recorded the observation of bodies similar to those described by Leishman in the blood from the spleen of patients suffering from kala-azar, and their universal association with the disease was soon established. Leishman's conclusion that the parasite seen by him and others was the involuted form of a trypanosome was not far from the mark, for next year, 1904, Rogers reported that he had cultivated the parasite in citrated blood outside the body, under which conditions it developed into what he at first thought to be a trypanosome. The main facts were subsequently confirmed by Leishman, but the flagellate form of the parasite turned out to be a variety of Leptomonas.

The significance of a flagellated form was not missed by Leishman, who pointed out the probability that this was the condition into which it would be found to develop in the body of some insect. The search for the insect transmitter of kala-azar has since continued, and from the work of the members of the Kala-azar Commission in India, it would appear to be a species of sand-fly (*Phlebotomus argentipes*). Successful transmission experiments by the bite of this insect have not, however, yet been accomplished, owing to the difficulty of finding a sufficiently susceptible experimental animal.

In 1904, J. H. Wright rediscovered parasites in "Delhi" boil similar to those described by Leishman, Cunningham's observations of 20 years before having been forgotten, and at the present time such parasites are known to be responsible for several diseases of men and animals. The controversy which took place over the zoological relationships of the parasites led to the conclusion that a new genus must be made for them, and, on the suggestion of Ross, the generic name, *Leishmania*, has been applied to the group.

Leishman devoted much of the time at his disposal for research after 1905 to the study of the spirochætes of relapsing fever. His earlier investigations were concerned with efforts to cultivate them and to distinguish the African from the European variety, the former being transmitted by the tick *Ornithodorus moubata*, and the latter by the body louse. All his efforts at cultivation, however, failed, and although Noguchi succeeded in 1912, Leishman was unable to repeat this. Neither by morphological characteristics nor by experiments on cross-immunisation was he able to satisfy himself of any difference between the two varieties. His experiments from 1908 onwards were directed to ascertain the life-history of the spirochæte after it was ingested by the tick, and the manner in which the infection was transmitted to man. As both monkey and mouse are susceptible to the African spirochæte, the answer to the questions could be supplied by experiments on these animals.

Koch, working in Africa in 1905, described the disappearance of the spirochætes in the stomach of the tick after a few days and their reappearance in the body fluid subsequently, and had also seen them in the ova, thus explaining the hereditary transmission of the infection in the tick. Leishman's first experiments were conducted at the temperature of a London laboratory, so that he was unable to confirm Koch's observations. He found it impossible to trace the spirochæte anywhere in the body of the tick later than the tenth day after feeding, and his search for spirochætes in the eggs laid by infected ticks proved negative. He noticed, however, that the spirochætes broke up into granules containing chromatin, and also noticed great numbers of similar granules in the bodies of some of the infected ticks.

The failure to see spirochætes, and the presence of abundant granules in ticks proved to harbour the infective agent, led Leishman to espouse the view previously enunciated by others, that these granules represented a stage in the life-history of the parasite. Later, when studying fresh tissues of infected ticks, he observed appearances which he interpreted as the growth of spirochætes from the granules. Although, on repeating his experiments at a temperature similar to that encountered in the tropics, he was able to confirm much of Koch's work, Leishman adhered to the opinion that fragmentation of spirochætes was not necessarily a degenerative process, but might represent a stage in the development of the parasite, and was probably an essential one for passing on the infection.

In his Horace Dobell Lectures to the Royal College of Physicians, in 1920, he presented the case for this opinion fairly, and stated his conclusions with moderation, pointing out that the work of Nicolle and others upon the transmission of *spirochæta recurrentis* by the body louse afforded collateral support. There is nothing inherently improbable in Leishman's view of the method, but the general consensus of opinion of parasitologists at the present time is against it.

Leishman's experiments on the means by which the infection passes from the tick to man failed to afford a decisive answer. He showed that the coxal fluid of the tick, which is often excreted by the insect whilst it is feeding, was infective, and he supposed that the parasite passed from this into the wound made by the bite, but he did not exclude a more direct method of implanting the infective agent by the insect during the act of sucking blood.

Another subject which occupied Leishman's attention was the etiology of blackwater fever. From a consideration of the epidemiology of the disease he arrived at the opinion that the view that this disease is merely a manifestation of malaria was untenable. He, therefore, took every opportunity to examine films of blood prepared from patients suffering from "blackwater," and described the occurrence therein of chromatin-staining inclusions. He inclined to believe that they represented either parasites or the reaction of the cell to the invasion of some infecting agent (chlamydozoa). Whether this be so can only be determined by further research. So far, no additional support has been forthcoming.

Leishman's biggest contribution to practical medicine was the work he did, from 1900 to 1915, to improve the efficiency and promote the practice of Wright's antityphoid inoculation in armies. When he returned to Netley Hospital in 1897, Wright had but recently introduced inoculation with a killed culture of the typhoid bacillus as a prophylactic measure. There was abundant sanction for the method from animal experiment, and its trial on troops in India under the supervision of its inventor had afforded encouraging results, but the best method of preparing a vaccine, its standardisation and the determination of the appropriate dosage still required much patient research.

As Wright's assistant, Leishman was engaged upon these researches, the first-fruits of which they published together in 1900. A method which gave promise of combating a disease which was taking a heavy toll of troops on foreign service, and which, in war, had often killed more men than the bullets of the enemy, appealed strongly to him and continued to occupy his attention for many years after the partnership with Wright ceased. The outbreak of the Boer War offered an opportunity for an extended trial of antityphoid inoculation. The permission of the War Office was obtained for the inoculation of troops as a voluntary measure, prior to their embarkation for South Africa, and the duty of preparing and standardising the vaccine for the troops was performed by Leishman.

The opinions formed as to the efficiency of antityphoid inoculation during the Boer War varied. Whilst the effect upon some units appeared to be most encouraging, in others the incidence of the disease upon inoculated and uninoculated showed little difference. The irregularity of results in different groups aroused the criticism of statisticians. It was argued that some other factors, such as variable efficiency of the vaccine used on different units, varying accuracy of diagnosis or in the records of cases, were producing an influence greater than that of inoculation. Such would appear to have been the case, for, at that time, paratyphoid diseases were not discriminated from typhoid, and no doubt a considerable proportion of the cases returned as typhoid during the Boer War were, indeed, not due to the *bacillus typhosus*. The unequal distribution of paratyphoid fevers in different units would alone be sufficient to account for the irregularities recorded.

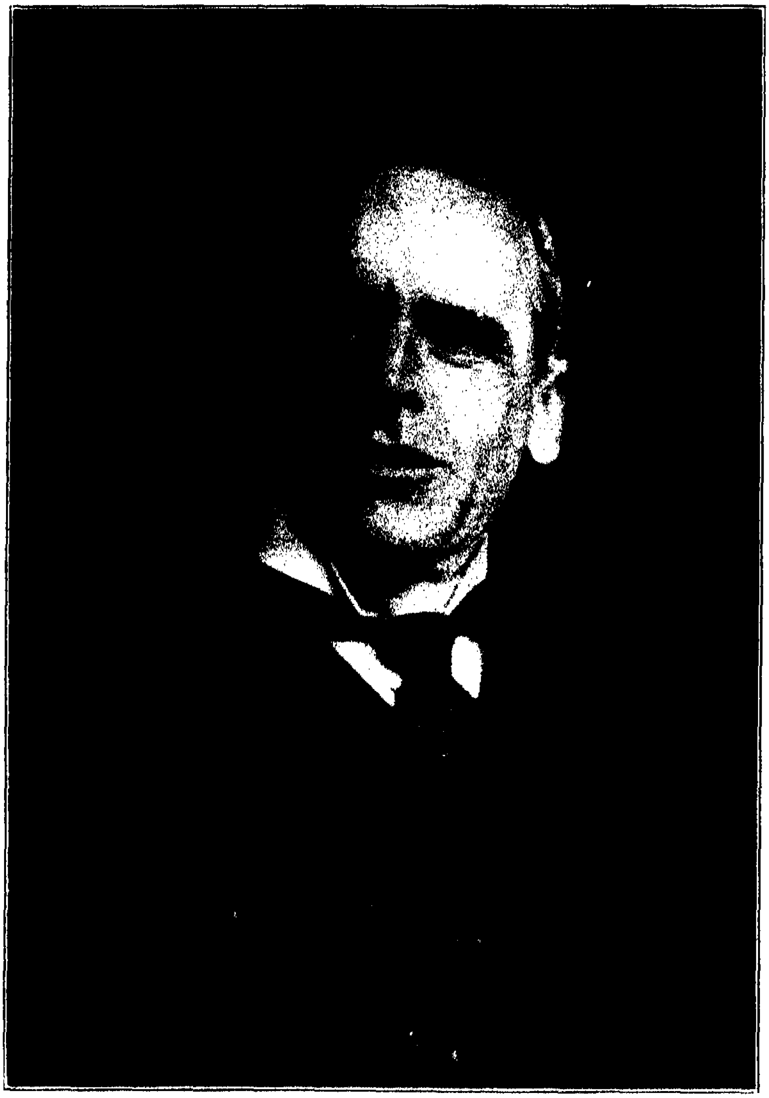
The lack of agreement upon the value of antityphoid inoculation led the Army Council, on the conclusion of the Boer War, to suspend the practice until further evidence had been collected. To secure this evidence they appointed a Departmental Committee, of which the present writer had the honour to be Chairman, to advise as to further action. After a study of the statistical and experimental evidence, this Committee presented an interim report, recommending that inoculation should be reintroduced as a voluntary measure, and that extensive experimental work should be undertaken with a view to the improvement of the system of inoculation.

A programme of research was drawn up by the Committee, and the duty of carrying out the proposed experiments devolved upon Leishman, who was a member of the Committee. The investigation proceeded in two directions: (1) the inoculation of approximately half of the individuals in all drafts proceeding to India and accurate recording of the subsequent history of both inoculated and uninoculated over a period of two years; (2) an experimental inquiry into the best method of preparing and administering the vaccine. To this end, the blood changes in animals following inoculation of the vaccine, as indicated by the amount of protective bodies aroused, were carefully and quantitatively studied in order to provide a standard method for comparison.

The inquiry occupied three years, and the credit for its successful conclusion was due to Leishman and his team of assistants. He was responsible for the details of its planning and its execution. In carrying out the programme of the Committee he showed himself to be possessed of scientific capacity of a high order and a rare administrative ability. As a result of these investigations the value of Wright's antityphoid inoculation was established beyond peradventure, a matter of deep concern when it is remembered that the medical history of all past campaigns has shown that the enteric group of diseases was as great a foe to armies on active service as the enemy.

When the European War broke out in 1914, the machinery for the immunisation of our Expeditionary Force was ready, but antityphoid vaccination was not compulsory in the British Army and, owing to the rapidity of mobilization, it was not possible to secure the inoculation of more than 25 per cent. of the troops before their embarkation for France in August, 1914. For military reasons, an attempt to remedy this defect could not be made until October, when the fighting had become localised on the Aisne. Meantime, typhoid broke out and began to spread. At this juncture, Leishman devoted himself to the task of persuading the men to be inoculated, and was extraordinarily successful. Owing to his strenuous and unremitting efforts, and those of his subordinates in the pathological service of the R.A.M.C., both at home and abroad, the proportion of inoculated soldiers on active service was increased in a couple of months to 80 per cent.; by the middle of 1915 it was 95 per cent., and by the end of the year 98 per cent. Closely following upon this successful campaign in favour of inoculation, the number of cases of typhoid fever diminished with nearly equal rapidity, and from the end of 1915 onwards was not a serious source of loss of military strength. Antityphoid inoculation does not make a man proof against the disease, but it reduces his chance of contracting it to about one-tenth, and this reduction in susceptibility was sufficient to prevent epidemic spread even under the conditions of trench warfare.

The invention of antityphoid inoculation we owe to Wright, but the successful immunization of our armies was, for the most part, due, directly and indirectly, to Leishman. By his experimental work the procedure was improved, by his



Ernest H. Starling

incessant propaganda the Army authorities were familiarised with the value of antityphoid inoculation, and, thanks to his tact and administrative ability, the many difficulties were overcome and the inoculation of millions of men was accomplished.

Had the casualties in the British Army from enteric fevers during 1914–18 been on the same scale as during the South African War, 900,000 of our soldiers would have been invalided, and 130,000 dead from them alone. If such a loss of man-power had occurred it must have influenced the result of the war. That the *total cases* were one forty-fifth and the *total deaths* one-hundredth of these figures is in part attributable to improvement in army sanitation, but there are good reasons for concluding that the greatest factor was the protection from epidemic extension afforded by the inoculation of almost the entire personnel of our armies. For this achievement, Leishman must be accounted to have been one of our most successful generals in the Great War.

C. J. M.

ERNEST HENRY STARLING—1866–1927.

Ernest Starling died on May 2nd, 1927, on board the *Ariguaing* shortly before reaching Kingston, Jamaica, whither he had gone on a short holiday for the benefit of his health. Of late years he had suffered from disabilities under which one of less heroic spirit could not have continued to work strenuously.

Starling was the eldest son of H. H. Starling, Clerk of the Crown at Bombay. The family of seven children had perforce to be educated in England. They therefore saw but little of their father and were brought up by their mother, an extraordinary woman, and it is to her influence by heredity and nurture that Starling owed his determination, mental alertness, and much of his charm. As the eldest boy, in the absence of a father he early acquired a sense of responsibility and capacity for managing his affairs, and was, in some respects, unusually mature for his years. He was educated at King's College School, and subsequently at Guy's Hospital.

When he began the study of the sciences preliminary to his medical education, his ambition was to be a physician, and it was not until a few years later that he contemplated the possibility of a life devoted to science and discovery.

Starling had, as a student, a brilliant academic career, about two-thirds of the academic honours available being secured by him. Physics and chemistry attracted him greatly, and the generalisation of biology, but it was when he came to study physiology that he found his real vocation, and he determined to become a physiologist, if such a career was economically possible.

After two years spent in the study of Anatomy and Physiology, he went for a few months to Heidelberg, and worked in Kühne's laboratory. He returned at the end of the year more than ever determined to become a physiologist. However, he had to put these aspirations aside for a while and apply himself to the study of practical medicine. Starling found great satisfaction and enjoyment in clinical work. The human side appealed to his sympathetic nature, and the immediate value of the application of knowledge to his practical mind.. After he became a physiologist he did not lose his interest in practical medicine, and if it had been possible to devote himself wholeheartedly to the study of medicine, as is now possible by the institution of full-time professorships, Starling would have been as happy investigating disease as in a physiological laboratory. Indeed, not many years ago he seriously considered accepting such a position.

He completed his medical studies in 1889, and graduated M.D. at London in 1890, qualifying for the gold medal in medicine. Immediately after graduation he became demonstrator at Guy's Hospital, and next year, joint lecturer in physiology with Golding Bird and Washbourne.

He was now confronted with two problems. The first was how he was to continue to subsist on one-third of a salary which was never intended to command the whole services of one physiologist; the second, how he was to secure a physiological laboratory to work and teach in. A Grocers' scholarship temporarily solved the first problem, and he determined that adequate accommodation and equipment for teaching and research in physiology should, ere long, by some means, be obtained. Meanwhile, he repaired to Schafer's laboratory at University College to carry on his researches. Bayliss was also working in the laboratory, and here began a scientific partnership which lasted on and off for thirty years. The two complemented one another in many respects. Whilst both possessed scientific imagination of the highest order, Starling was more ardent and forceful, eager to translate ideas into action, but rather bored with details of technical method. He had never been interested in doing things with his hands, except climbing, although later he became a most beautiful and dexterous operator. Bayliss was the philosophical student, calm, with better critical judgment. He read widely and had a wonderful knowledge of scientific literature, was an excellent mechanic, and found enjoyment in the development of the technical methods of research. How fruitful this partnership was will be seen from the account below of Starling's scientific work.

The equipment at Guy's Hospital was steadily improved, and Starling and Bayliss did their work on the innervation of the heart together there, and Starling his on the factors controlling transudation and lymph flow, and also his earlier work on urinary secretion. By 1895 Starling had planned a really good physiological institute for Guy's Hospital, and secured the assent of the authorities to its erection. These laboratories were completed in 1897 and were, at that time, the best in London. He did not enjoy their amenities for long, as in 1899 he was appointed to the Jodrell chair of Physiology at University College.

It was a wrench to leave his new laboratories, to the planning of which he had devoted so much time, but he felt that the "atmosphere" at University College was more favourable to the realisation of his ideals. The College was, at the time, in low water financially, but he firmly believed that this temporary embarrassment could be removed, and that it need not be long before the medical sciences at University College were appropriately housed and equipped. Starling dreamed dreams, but he was also a man of action. He gave all the money he possessed to improve the equipment of the existing physiological laboratories and threw himself whole-heartedly into projects for eliminating the deficit in the College funds, and to raise the money for an Institute of Medical Sciences.

His faith was justified, and in 1909 the first instalment of his scheme came to fruition, when the present Institute of Physiology was opened, the cost of which was largely defrayed by money raised by his personal efforts. Starling's objective was not merely a new physiological school, in which he was naturally more particularly interested. He wanted all the medical sciences at University College to have the advantages of more commodious and nobler buildings. His original scheme included accommodation for anatomy and pharmacology as well as physiology, with a central library, pathology being well housed in the new Clinical School. At first only the physiological section was built, but a few years ago the erection of the whole Institute, as originally contemplated, was rendered possible by a generous gift from the Rockefeller Foundation, the directors of which were desirous of devoting funds in the interest of medical science in London. That they chose this particular means of fulfilling their aims was largely, if not entirely, due to Starling, and the noble Institute of Medical Sciences with which University College is now endowed is a fine material monument to the memory of one who not only helped to build up a great school of physiology in London and obtained an appropriate habitation for it, but also was as unsparing in his efforts to secure similar advantages for the other medical sciences.

In 1922 Starling retired from the Jodrell chair, on his appointment to a Foulerton Research Professorship of the Royal Society. This was the first of the Royal Society's professorships to be founded, and Starling was the first holder. This happy precedent has been followed and there are now five research professorships. He still continued to work at University College, but was relieved of all administrative duties and all teaching except that of a small band of research pupils. His laboratories continued to be a centre of great activity, and a limited number of distinguished young physiologists from Great Britain and abroad enjoyed the advantage of working in close communion with one of the greatest masters of experimental physiology until April, 1927.

Starling was elected a Fellow of the Society in 1899. He was Royal Medallist in 1913. He thrice served on the Council and was chairman of the Physiology and Medical Sciences Committee at the time of his death.

STARLING'S PHYSIOLOGICAL RESEARCHES.

Starling's interest in physiology was general, but the subjects for investigation which particularly attracted him were those physiological processes which seemed capable of interpretation in terms of chemistry and physics.

He was always, from his student days, fascinated with the problem of the heart and the adjustment of its action to varying conditions of the body, and his first paper, written with Bayliss in 1891, was on the electromotive phenomenon of the mammalian heart. Waller had recently studied the electrical variation of the excised heart, and also of the heart *in situ*, by leading off from the neighbourhood of the apex and base respectively. They believed that by photographing the movements of the capillary electrometer, connected with electrodes placed in different positions on the naked heart in the living animal, much might be learnt of the nature of the cardiac contraction ; in fact, that a new method of observation was at the disposal of the investigator. At that time, any sort of muscular continuity between the auricles and ventricles was denied, and the view that conductivity was due to some nervous network supplying the fibres was in favour.

They next explored the separate action of the vagi and accelerator nerves on the auricles, on the ventricles, and on the conducting power of the auriculo-ventricular junction in the mammal. The effects of these nerves on the hearts of frogs and tortoise had been previously studied by Gaskell and Heidenhain. Bayliss and Starling completed the story, showing that there was no essential difference between the hearts of mammals and cold-blooded animals, and that the vagus depresses conduction in auricle, auriculo-ventricular junction and ventricle, and that the accelerator nerves had the opposite effects on all three structures.

Two other important papers dealing with the mechanism of the circulation were published by Bayliss and Starling at this time. One was an exhaustive study of the simultaneous changes in the arterial and venous pressures of various regions of the body under a great variety of experimental conditions. The results showed the universal applicability of the principle of the circulation worked out by Ludwig. The other was an analysis of simultaneous pressures in the aorta and ventricles of the heart *in situ.* They used a continuous photographic record of the changes in volume of a small air-space at the end of a capillary glass tube connected with the aorta and ventricle respectively. This method being almost free from inertia and aperiodic, they succeeded in obtaining a true record of the rapid variations occurring in the ventricle and aorta and the precise relation of these to one another. Their measurements have been the standard of reference ever since.

In 1892 Starling went to work with Heidenhain in Breslau. Heidenhain had distinguished two kinds of lymphagogues, and under his inspiration Starling set to work to make a more detailed analysis of the effects of one of them, peptone. In summarising his results he adopted the interpretation of Heidenhain that the experimental facts concerning lymph formation could not be explained by filtration, and that it was necessary to suppose a selective activity on the vessel wall. On returning to England he continued to work at the problem of lymph formation and repeated all of Heidenhain's experiments. He was able to confirm his facts but came to doubt the correctness of his interpretation. He searched for evidence of lymph-secretory nerves, but found that the nervous system could only influence lymph-flow by altering vascular conditions. After years of experimenting he came to the conclusion that it was unnecessary to suppose a secretory activity of the endothelium, if some other factor existed to balance the hydrostatic pressure in the capillaries.

In 1896, Starling discovered that the missing factor required to afford a complete interpretation of the phenomena was the osmotic pressure of the colloids, to which the walls of the capillaries are relatively impermeable. It had hitherto been supposed that the osmotic pressures of proteins, being so insignificant compared to those of salts, must be of no account in physiological processes. The reverse is indeed the case, because it is only to the proteins that the membrane is impermeable. He therefore set to work to measure the osmotic pressures of the proteins in serum and found them to be, though small, of the order of magnitude of the capillary pressure. The problem was solved. The hydrostatic pressure and the osmotic pressure supplied the balance of forces necessary to explain the experimental observations.

Starling's work on lymph formation occupied five years, and is of the best he did. After long-continued and difficult experimentation, combined with observation of the highest order of accuracy, this hitherto obscure but fundamentally important region of physiology was finally illuminated by his dexterous experimentation and triumphant imagination.

Bayliss and Starling next embarked together upon an investigation on the intestinal movements and the innervation of the small and large intestine. The work was begun at Guy's Hospital and completed at University College.

When they undertook this study, the nerve supply to the small and large gut had been carefully determined by Langley and Anderson, but of the working of the neuromuscular mechanism there were many discrepancies as to fact and opinion. After eighteen months' careful experimenting, with appropriate recording methods devised for the purpose, they were able to reduce the previous chaos to order and to summarise the main facts concerning intestinal movements in a few simple statements. (1) That peristaltic contractions are true co-ordinate reflexes carried out by the local nervous mechanism and independent of the connexion with the central nervous system. (2) Local stimulation of the gut produces excitation above, inhibition below. (3) Besides the local mechanism, every part of the gut is subject to the control of the central nervous system through the splanchnics and vagi, the former being inhibitory and the latter containing both augmenting and inhibitory fibres.

This was as far as understanding of the matter progressed until Cannon introduced the method of observation by means of X-rays in an animal fed upon a bismuth meal. They next tried to find out how pancreatic secretion was brought about when food entered the duodenum. The discoveries of Pawlow had determined the order of events in gastric secretion and their co-ordination through the agency of the nervous system, but although he had found that no secretion from the pancreas occurred until the acid chyme reached the duodenum, just how pancreatic secretion was called forth had baffled this great experimenter and his pupils. Popielski had determined that the introduction of acid into the upper part of the small intestine caused secretion from the pancreas, notwithstanding previous section of the vagi and sympathetic, or even complete extirpation of the solar plexus. He concluded, therefore, that secretion must be brought about by means of some local nervous apparatus.

Bayliss and Starling started their investigations with the idea of deciding where this peripheral nervous mechanism was. They verified all the facts stated by the Russian physiologists, but they were unsuccessful in proving the existence of any nervous mechanism controlling pancreatic secretion. Nor could they discover how secretion was brought about until they made the crucial experiment which led to the discovery of secretin. I happened to be present at their discovery. In an anæsthetised dog, a loop of jejunum was tied at both ends, and the nerves supplying it dissected out and divided, so that it was connected with the rest of the body only by its blood-vessels. On the introduction of some weak hydrochloric acid into the duodenum, secretion from the pancreas occurred and continued for some minutes. After this had subsided, a few cubic centimetres of acid were introduced into the enervated loop of jejunum. To our surprise, a similarly marked secretion was produced. I remember Starling saying, "Then it must be a chemical reflex." Rapidly cutting off a further piece of jejunum, he rubbed its mucous membrane with sand in weak hydrochloric acid, filtered and injected it into the jugular vein of the animal. After a few moments, the pancreas responded by a much greater secretion than had occurred before.

Bayliss and Starling followed up their discovery in many important directions which space forbids me to mention. A method of obtaining natural pancreatic juice was now available, and they made full use of their opportunities to study trypsinogen and its conversion into trypsin by enterokinase. Starling was also moved by them to much constructive thought and further research on the chemical integration of the bodily functions generally. He proposed the name " hormones " or chemical messengers for all such active principles formed in one part of the body and distributed by the circulation to excite the normal functioning or stimulation of growth of other parts, and this term has become generally used.

In 1909, Starling returned again to the investigation of the action of the heart. He and his pupil Kawa had been attempting to dissociate the effects of asphyxia on the circulation into those due to diminished oxygen and increased carbonic acid tension respectively. They used the " spinal animal," that is, one in which the brain above the pons has been destroyed. They obtained some interesting information, but the observations were difficult to interpret without being able to separate the effects of alteration in the gaseous composition of the blood upon the heart itself.

To satisfy these requirements the heart must be isolated from the rest of the body and at the same time fed with a constant supply of perfectly oxygenated blood ; it must be working under mechanical conditions, all of which are completely under the control of the experimenter. This was accomplished by a device, now famous, known as Starling's heart-lung preparation.

This experimental device did a great deal more than serve the purpose for which it was originally designed and the years immediately succeeding the development of this method of studying cardiology were the most productive, from the point of view of scientific output, in Starling's career. He was surrounded by enthusiastic and able pupils drawn from all over the world. He had plenty of problems for them to attack, with every prospect of a reasonable reward for their efforts. Starling was unsparing in helping them towards the solution of the problem allocated to each, often performing the more difficult parts of the experiments himself and afterwards writing their papers for them.

It is only possible to indicate a few of the more fundamental facts established by this happy band of discoverers until it was scattered by the outbreak of war in 1914. Detailed accounts will be found in the publications from his laboratory between the years 1910 and 1915.

In the first case, the effects of variations in the tensions of oxygen and carbon dioxide in the blood upon the diastolic volume and output of the heart, on its capacity for work, and on the flow through the coronary arteries, were determined. The heart was found to have an astounding power of utilising the oxygen in the blood. When an isolated heart was fed with blood from an asphyxiated animal, the heart removed all but traces of oxygen. The conditions controlling the rate of the heart-beat were studied, and the only influences found to modify the rate of the isolated heart were temperature, and adrenalin. The maximum output of the heart was measured and found to be three litres a minute for a dog's heart weighing 50 gm. Observations were also made upon the energetics of the heart by Starling and Evans, and afterwards by Evans, who determined the oxygen used per unit of work done. The respiratory quotients of the normal and diabetic heart were ascertained, and from those two sets of observations the efficiency of the heart as a machine working under various loads was calculated to reach 20 per cent.

The experiments upon the flow of blood through the coronary arteries showed that this flow was primarily dependent upon the arterial pressure, but that dilatation of the coronary system occurred when the carbon dioxide tension in the blood increased, when adrenalin was added, and most markedly when some metabolites, the product of the heart's own activity, were added to the blood circulating.

The most significant of all these discoveries about the heart's action were those made by Starling and his pupils, Knowlton, Patterson and Piper, demonstrating that, within wide limits, the output of the isolated heart is independent of the rate of its beat, of the arterial pressure and of the temperature, but controlled by the inflow. This living pump, isolated from all connexions with the nervous system, is able, within wide limits, to increase its output automatically, according to the inflow, whatever the work involved.

The mechanism of this self-regulation was sought for during two years and after much ingenious experimentation, in which, later, Vischer took part, Starling and his collaborators demonstrated that there was a direct proportion between the diastolic volume of the heart and the energy set free in the following systole. The importance of this observation derives from the fact that the diastolic volume and the length of the muscle fibres are closely related, and therefore, that cardiac muscle obeys Hill's law for skeletal muscle, that the energy set free on passing from the resting to the contracted state depends upon the length of the muscle fibres.

This simple formula serves to explain the whole behaviour of the isolated mammalian heart, for, owing to this property of its muscle, if the heart fails to empty itself at one of its contractions it starts the next at greater physiological advantage.

Their researches on the heart had reached this interesting point when the European War occurred. For a few months Starling carried on his professorial work at University College, but became more and more unsettled. He wanted to go and fight. Persuaded, if not convinced, that this was not the most suitable manner in which to satisfy his strong tribal instincts, he joined the

R.A.M.C. as a Captain and was for some time a medical officer at the Herbert Hospital. Later, as the scientific resources of the country were mobilised, he was made Director of Research at the R.A.M.C. College and was busy experimenting with defensive methods against poison gases. In this he rendered invaluable service to his country. No one could have been better to control the experimental side of these researches. In 1916 he was promoted to Lt.-Colonel and sent as scientific adviser for anti-gas services to Salonika and to the Italian front. In 1917 he resigned his commission, deeming that he could be of greater service as a civilian. On his retirement he was appointed a C.M.G.

At that time food shortage seemed most likely to decide the issue, and Starling became Chairman of the Royal Society Food Committee, which was responsible for advising the Government on scientific matters fundamental to the rationing of our food supplies. He was subsequently scientific adviser to the Ministry of Food, and British scientific delegate on the Inter-Allied Food Commission. In all these capacities Starling rendered yeoman service. He soon had a mastery of the necessary facts, and he was by nature and training able to marshal them comprehensively and arrive at definite conclusions. At the same time, he realised to the full that in applying physiological principles to administrative action, the economic side of the problem was of equal or greater importance; consequently, he impressed all those statesmen and officials with whom he had to deal as one possessing, not only great knowledge, but sound judgment. It is doubtful whether any other of our physiologists could have served us so well.

For a while, after the War, Starling's work was seriously curtailed owing to ill health, which finally necessitated a serious surgical operation. However, in 1920, he was back again at work, with Anrep, on the central and reflex regulation of the circulation, using a cross-circulation method, built up on his heart-lung preparation in which the circulation through the brain of the animal was entirely under the control of the experimenter, while the animal's own heart supplied the rest of its body. By this ingenious experimentation, they were able to ascertain how the circulation in other parts of the body is modified in accordance with changes in the circulation in the brain.

After this Starling ceased for a while his experiments on the heart and turned his attention to other matters, and during the last five years of his life was mostly occupied in attempting to solve some of the many perplexities of renal secretion. It was not for the first time that he turned his attention to this subject. His experiments in 1899 had indicated that the glomerular epithelium was a simple filtering membrane, resembling a film of gelatine in permeability, whereas active secretion took place in the epithelium of the tubules. He abandoned the subject at that time, because he could see no chance for further advance until experiments could be made upon a functionally active kidney separated from the body and with some of the many factors controlling urinary secretion under experimental control.

Many attempts to achieve the necessary experimental conditions had failed, but the heart-lung preparation afforded a means by which any isolated organ might be fed with arterial blood of known composition at any desired pressure, rate of flow, and temperature. It was thus possible to study the functions of an organ apart from nervous influences and from the chemical influences which arose in consequence of modifications in the blood caused by other organs of the body. After numerous attempts, Starling and Verney succeeded in maintaining the isolated kidney in such a condition that it would secrete abundant urine.

By this method, which demands extraordinary experimental skill, Starling has opened a new chapter on the physiology of renal secretion. Already, many new facts, and others which were previously only matters of surmise, have been discovered and established. His observations with Verney and in collaboration with Eichholtz have shown that the glomeruli filter from the blood plasma its non-protein constituents, and that by using hydrocyanic acid to suspend tubular activity, a pure glomerular filtrate is obtained from the ureter. Also that, whilst urea and sulphate are secreted by the tubule cells, water, chloride, bicarbonate and glucose are re-absorbed by the tubule cells from the glomerular filtrate. Pituitrin increases the amount of chloride and decreases the amount of water eliminated.

The influence of the pituitary gland upon the secretion of the kidney was particularly studied by Starling's pupils, Eichholtz and Bruhl. Their experiments suggest that the inability of the isolated kidney to secrete inorganic phosphate is due to the absence of the pituitary hormone. If this be so, it is another discovery of a chemical correlation of the body, for which Starling is largely responsible.

These researches on the isolated kidney were in full swing in April when Starling left for the holiday which was long overdue. They were affording most promising results, and doubtless, had he been spared to continue them, he would, with his unrivalled experimental skill, ultimately have succeeded in clarifying our knowledge of urinary secretion, as he had laid bare the principles involved in the self-adjustments of the heart to physiological requirements.

Starling's influence upon physiology is not confined to his own contributions to knowledge. He was also a great exponent of the science he loved so well. His "Principles of Human Physiology" is widely read on both sides of the Atlantic and generally regarded as the best exposition of the subject in the English language. The more limited audience of his own class rooms found in him a stimulating teacher whose enthusiasm was infectious. His research pupils idolized him. To everyone, provided he were a serious inquirer after truth, he extended his help, encouragement and friendship.

Starling was the recipient of many academic honours. Honorary degrees were conferred upon him by the Universities of Dublin, Sheffield, Cambridge, Breslau, Strasburg and Heidelberg. He received the Baly Medal in 1907 and the Royal Medal in 1913. No account of his career would be complete without allusion to the part played in it by his wife. In 1891 he married Florence Wooldridge, the daughter of Sir Edward Sieveking. They were inseparable companions. With unselfish devotion she helped him more than will ever be known. They were fellow conspirators in all his projects, and for many years she performed for him the functions of an efficient secretary.

What place amongst the great discoverers in medical science should be allotted to Starling must be left to the judgment of posterity, but it will be generally conceded by his contemporaries that he was one of the foremost physiologists of our time, and that no one since Harvey has so greatly advanced our knowledge of the action of the heart.

C. J. M.

INDEX TO VOL. CII. (B)

Air-resistance to a runner (Hill), 380.
Anniversary Address, Nov. 30, 1927 (Rutherford), 239.
Apple fruit, study of course of fungal invasion of (Gregory and Horne), 427, 444.
Assimilation and respiration, vegetable, experimental researches on (Maskell), 467, 488.

Bacteria, alcohol-soluble proteins in (Goadby), 137.
Barnwell (J. B.) See Richards and Barnwell.
Bismuthyl derivatives of organic hydroxy-acids, therapeutic action of (Browning and others), 1.
Brambell (F. W. R.) The Development and Morphology of the Gonads of the Mouse. Part II.—The Development of the Wolffian Body and Ducts, 206.
—— Fielding (U.) and Parkes (A. S.) Changes in the Ovary of the Mouse following Exposure to X-Rays. Part IV.—The Corpus Luteum in the Sterilized Ovary and some Concluding Experiments, 385.
Briggs (G. E.) A Consideration of some Attempts to Analyse Growth Curves, 280.
Brown (T. G.) Absence of a Linear Relationship between Graded Simple Reflex Flexions and the Relaxations thereof evoked by a Constant Extension-producing Stimulus, 143; Absence of a Linear Relationship between the Reflex Flexor Shortenings evoked by a Graded Series of Flexion-producing Stimuli and the "Inhibitory" Lengthenings of a Constant Extension Reflex, 150; The Relation of the Magnitudes of Remaining Reflex Shortening in Two Antagonistic Muscles during Compound Stimulation, 159.
Browning (C. H.) (and others) The Therapeutic Action of some Bismuthyl Derivatives of Organic Hydroxy-Acids, 1.
Bury (F. W.) See Sprawson and Bury.

Canti (R. G.) and Spear (F. G.) The Effect of Gamma Irradiation on Cell Division in Tissue Culture *in vitro*, 92.
Carleton (H. M.) and Florey (H.) The Mammalian Lacteal, its Histological Structure in Relation to its Physiological Properties, 110.
Cell division, effect of gamma irradiation on (Canti and Spear), 92.
Cell, microchemistry of—chromatin content (Ludford), 397.
Cerebral cortex, responses of motor area of (Cooper and Denny-Brown), 222.
Cohen (J. B.) See Browning and others.
Cooper (S.) and Denny-Brown (D.) Responses to Stimulation of the Motor Area of the Cerebral Cortex, 222.
Croonian Lecture (Spemann), 77.

Denny-Brown (D.) See Cooper and Denny-Brown.
Dickinson (S.) Experiments on the Physiology and Genetics of the Smut Fungi—Seedling Infection, 174.

Einthoven (W.) Obituary notice of, v.
Eltringham (H.) On the Production of Silk by Species of the Genus *Hilara* Meig. (Diptera), 327.
Enamel, organic content of human (Sprawson and Bury), 419.

Fell (H. B.) See Strangeways and Fell.

Fielding (U.) See *Brambell and others.*

Findlay (G. M.) Immunological and Serological Studies on the Viruses of Fowl-pox and Vaccinia, 354.

Fisher (R. A.) Triplet Children in Great Britain and Ireland, 286.

Florey (H.) See Carleton and Florey.

Fowl-pox and vaccinia, studies on viruses of (Findlay), 354; (Ludford), 406.

Furusawa (K.), Hill (A. V.) and Parkinson (J. L.) The Dynamics of "Sprint" Running, 29; The Energy used in "Sprint" Running, 43.

Gamma irradiation and cell division (Canti and Spear), 92.

Goadby (Sir K.) Bacterial Proteins.—The Presence of Alcohol-soluble Proteins in Bacteria, 137.

Gonads of mouse, development and morphology (Brambell), 206.

Gray (P. H. H.) The Formation of Indigotin from Indol by Soil Bacteria, 263.

Gregory (F. G.) The Differential Effect of the Ions of Three-salt Solutions on the Growth of Potato Plants in Sand Culture, 311.

—— and Horne (A. S.) A Quantitative Study of the Course of Fungal Invasion of the Apple Fruit, and its Bearing on the Nature of Disease Resistance.—Part I. A Statistical Method of Studying Fungal Invasion, 427; Part II. The Application of the Statistical Method to certain Specific Problems, 444.

Growth of potato plants, effect of 3-salt solutions on (Gregory), 311.

Guanidins, action on skin melanophores of frog (Ochoa), 256.

Hamm (A. H.) On the Epigamic Behaviour of *Hilara maura*, Fab., and two Allied Species, 334.

Harrison (J. W. H.) A Further Induction of Melanism in the Lepidopterous Insect, *Selenia bilunaria*, Esp., and its Inheritance, 338; Induced Changes in the Pigmentation of the Pupæ of the Butterfly *Pieris napi* L., and their Inheritance, 347.

Hilara, production of silk by (Eltringham), 327; (Hamm), 334.

Hill (A. V.) The Air-Resistance to a Runner, 380.

—— See Furusawa (K.), etc.

Hill (L.) Measurement of the Biologically Active Ultra-violet Rays of Sunlight, 119.

Hindle (E.) and Patton (W. S.) Transmission Experiments with Chinese Kala Azar, 63.

Horning (E. S.) and Petrie (A. H. K.) The Enzymatic Function of Mitochondria in the Germination of Cereals, 188.

Indigotin, formation from indol by soil bacteria (Gray), 263.

Kala azar, transmission experiments with (Hindle and Patton), 63.

Lacteal, mammalian, structure and properties (Carleton and Florey), 110.

Lathyrus, linkage groups and chromosomes (Punnett), 236.

Leishman (Sir W.) Obituary Notice of, ix.

Lister (J. J.) Obituary Notice of, i.

Ludford (R. J.) Studies in the Microchemistry of the Cell.—I. The Chromatin Content of Normal and Malignant Cells, as demonstrated by Feulgen's "Nuclealreaktion," 397 Cytological Studies on the Viruses of Fowl-pox and Vaccinia, 406.

Lymphatics, distribution defined by fatty acid compounds (Smith and Rettie), 102.

Maskell (E. J.) Experimental Researches on Vegetable Assimilation and Respiration. XVII.—The Diurnal Rhythm of Assimilation in Leaves of Cherry Laurel at "Limiting" Concentrations of Carbon Dioxide, 467; XVIII.—The Relation between Stomatal Opening and Assimilation.—A Critical Study of Assimilation Rates and Porometer Rates in Leaves of Cherry Laurel, 488.

Melanism in *Silenia bilunaria* (Harrison), 338.

Mitochondria in germinating cereals, enzymatic function (Horning and Petrie), 188.

Obituary Notices of Fellows deceased:—
  Einthoven, W., v.
  Leishman, W. B., ix.
  Lister, J. J., i.
  Starling, E. H., xvii.

Ochoa (S.) The Action of Guanidins on the Melanophores of the Skin of the Frog (*Rana temporaria*), 256.

Organizers in animal development (Spemann), 177.

Ovary of mouse, X-ray changes in (Brambell and others), 385.

Parkes (A. S.) On the Occurrence of the Œstrous Cycle after X-Ray Sterilisation. Part IV.—Irradiation of the Adult during Pregnancy and Lactation; and General Summary, 51.

—— See Brambell and others.

Parkinson (J. L.) See Furusawa (K.), etc.

Patton (W. S.) See Hindle and Patton.

—— and Hindle (E.) The North Chinese Species of the Genus Phlebotomus (Diptera, Psychodidæ), 533.

Petrie (A. H. K.) See Horning and Petrie.

Phenolsulphonephthalein, secretion of, by renal tubule (Richards and Barnwell), 72.

Phlebotomus, N. Chinese species (Patton and Hindle), 533.

Pigmentation, induced, of pupæ of *Pieris napi* (Harrison), 347.

Punnett (R. C.) Linkage Groups and Chromosome Number in Lathyrus, 236.

Reflex flexions and relaxations, absence of linear relationship (Brown), 143.

Reflex flexor shortenings and inhibitory lengthenings of extension reflex (Brown), 150.

Reflex shortening in antagonistic muscles during compound stimulation (Brown), 159.

Rettie (T.) See Smith (J. L.) and Rettie.

Richards (A. N.) and Barnwell (J. B.) Experiments concerning the Question of Secretion of Phenolsuplhonephthalein by the Renal Tubule, 72.

Rutherford (Sir E.) Presidential Address, Nov. 30, 1927, 239.

Silk, production of, by *Hilara* (Diptera) (Eltringham), 327; (Hamm), 334.

Smith (J. L.) and Rettie (T.) The Distribution of Lymphatics defined by Fatty Acid Compounds developed in the Autolysis of their Contents, 102.

Smut fungi, physiology and genetics (Dickinson), 174.

Spear (F. G.) See Canti and Spear.

Spemann (H.) Croonian Lecture:—Organizers in Animal Development, 177.

Sprawson (E.) and Bury (F. W.) On the Chemical Evidences of the Organic Content of Human Enamel, 419.

"Sprint" running, dynamics of (Furusawa and others), 29; Energy used in (Furusawa and others), 43.

Starling (E. H.) Obituary Notice of, xvii.
Strangeways (T. S. P.) and Fell (H. B.) A Study of the Direct and Indirect Action of X-Rays upon the Tissues of the Embryonic Fowl, 9.

Triplet children in Great Britain and Ireland (Fisher), 286.

Ultra-violet rays, measurement of biological activity (Hill), 119.

X-Ray changes in ovary of mouse (Brambell and others), 385.
X-Ray sterilization and œstrous cycle (Parkes), 51.
X-Rays, action upon tissues of embryonic fowl (Strangeways and Fell), 9.

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14.11.55		
16.12.57		
20.4.65		
28.12.79 L.L.		